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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/82, 15/12, A01N 63/02, A01H 5/00 | A1

(11) International Publication Number: WO 98/0686

(43) International Publication Date: 19 February 1998 (19.02.9

(21) International Application Number: PCT/EP97/04438

(22) International Filing Date: 13 August 1997 (13.08.97)

(30) Priority Data:
60/023,940 14 August 1996 (14.08.96) US

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, B BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, G GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, L LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, N PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, T TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (G KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, A BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, B CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, N PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, G ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending t claims and to be republished in the event of the receipt amendments.

(54) Title: PEPTIDE WITH INHIBITORY ACTIVITY TOWARDS PLANT PATHOGENIC FUNGI

(57) Abstract

Lactoferricin has been found to have antifungal activity against plant pathogens. Genetic constructs comprising a synthetic ge for bovine lactoferricin are prepared and used to provide transgenic disease-resistant plants. A method of transforming plants to expre lactoferricin which is expressed in plants to provide disease resistance to those plants is also described.

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Peptide with Inhibitory Activity Towards Plant Pathogenic Fungi

This invention relates to a method of controlling plant pathogens by a peptide which is provided by genetically modifying the plant to produce the peptide, and to genes and plants useful in that method. In particular, this invention relates to a lactoferricin and its use in controlling plant pathogens.

Numerous fungi and bacteria are serious pests of economically-important agricultural crops. Various methods of controlling diseases in plants have been used with varying degress of sucess. One method has been to apply an antimicrobial chemical to crops. This method has numerous, art-recognized problems. Alternatively, a more recent method involves the use of biological control organisms ("biocontrol") which are natural competitors or inhibitors of the pest organism. However, it is difficult to apply biocontrol to large areas, and even more difficult to cause those living organisms to remain in the treated area for an extended period of time. More recently, techniques in recombinant DNA have provided the opportunity to insert into plant cells cloned genes which express antimicrobial compounds. However, this technology has given rise to concerns about eventual microbial resistance to well-known, naturally occurring antimicrobials. Thus, a continuing need exists to identify naturally occurring antimicrobial compounds which can be formed by plant cells directly by translation of a single gene.

It is particularly interesting to identify novel peptides and DNA sequences encoding said peptides since peptides having antipathogenic and antimicrobial activity are already known in the art. For example, there are many so-called lytic peptides from animal, plant, insect, and microbial sources including the mammalian defensins, cecropins, thionins, mellitins, insect defensins, and the like.

Another class of peptide with antipathogenic activity is represented by hydrolytic enzymes such as chitinase and β-1,3-glucanase which are known to inhibit fungal growth (Schlumbaum, *et al.*, 1986; Mauch, *et al.*, 1988).

Enzymatic hydrolysates of the milk protein lactoferrin are also shown to have antimicrobial properties. Lactoferrin is an iron binding glycoprotein present in most biological fluids of mammals, including milk, tears, and mucous secretions (Brock, J., Arch. Dis. Child. 55: 417 (1980); Bullen, J. Rev. Infect. Dis. 3: 1127 (1981)). Lactoferrin has

been associated with the promotion of cell growth, regulation of hematopoiesis, and protection against microbial infection and immune modulating properties *in vitro*. Little is known, however, about the function of lactoferrin in vivo. Extracellular lactoferrin has been reported to have antibacterial and antiviral activities. Work with bovine lactoferrin has found that the antimicrobial activity of an enzymatic hydrolysate generated by digestion with porcine pepsin is stronger than that of the whole protein against an Escherichia coli lysate (Tomita, *et al.*, J. Dairy Sci. 74:4137-4142 (1991).

The antifungal activity of lactoferrin has also been demonstrated in humans. The susceptibility of *Candida albicans* to inhibition and inactivation by lactoferricin B has been examined by Bellamy *et al.*, Med. Microbiol. Immunolog. 182: 97-105 (1993). Its effect is lethal, causing a rapid loss of colony-forming capability, suggesting that the active peptides of lactoferrin could potentially contribute to the host defense against *C. albicans*.

Human lactoferrin cDNA was recently expressed in tobacco suspension cells to test the antibacterial activity of lactoferrin in plants. Transgenic calli produced a single peptide of 48 kD which is significantly smaller than the full length lactoferrin protein. Total protein extracts made from transgenic tobacco callus exhibited much higher antibacterial activity than commercially available purified lactoferrin as determined by the decrease of colony forming units when tested with four phytopathogenic species of bacteria. The full length lactoferrin was never detected in transgenic calli implying that the lactoferrin gene product had undergone posttranslational processing. Since a human lactoferrin gene was expressed in *Aspergillus nidulans* with the production of large amounts of full-length lactoferrin, it was theorized that full length plant-produced lactoferrin does *not* undergo proper folding and the unfolded part is degraded. The truncated protein exhibiting antibacterial activity in calli was identified as the amino- or carboxy-terminal end of the protein.

The present invention provides an enzymatic hydrolysate of lactoferrin that can be safely expressed in plants to provide disease resistance. Genetic constructs comprising a synthetic gene for lactoferricin are prepared and used to provide transgenic disease-resistant plants. A method of transforming plants to express lactoferricin which is expressed in plants to provide disease resistance to those plants is also described.

Figure 1: Plasmid pCIB7703, containing the lactoferricin B gene (from position 1995-2079) under control of the ubiquitin promoter (15-1995) and the nos terminator (2079-2339).

Figure 2: Plasmid pCIB7704, similar to pCIB7703 with the ubi-SynPat-nos selection cassette (from position 15-2845).

Figure 3: Plasmid 7705, containing the ubi-SynPat-nos -- ubi-lactoferricin B-nos fragment in an NptII containing plasmid.

Figure 4: Plasmid 7706, containing the ubi-lactoferricin B-nos fragment in an NptII containing plasmid.

The present invention utilizes lactoferricins, which are enzymatic hydrolysates of lactoferrins having antipathogenic activity and corresponding to the N-terminal region of a lactoferrin. Lactoferrins are proteins produced in mammalian milk which have structural and functional homologies among the different species, although there is some variation in the precise amino acid sequence from species to species. For use in the present invention, the preferred lactoferricins include lactoferricin B (bovine lactoferricin) or lactoferricin H (human lactoferricin). Lactoferricins are now found to inhibit the growth of a number of agronomically important pathogens and to be capable of expression in plants thereby conferring disease resistance.

Lactoferricin B (or bovine lactoferricin) is a peptide of 25 amino acids long:

Phe-Lys-Cys-Arg-Arg-Trp-Gln-Trp--Arg-Met-Lys-Lys-Leu-Gly-Ala-Pro-Ser-Ile-Thr-Cys-Val-Arg-Arg-Ala-Phe (SEQ ID NO:1)

having exact homology with the N-terminus of the whole bovine lactoferrin sequence.

Lactoferricin H (or human lactoferricin) corresponds to amino acids 1-33 of human lactoferrin, and is thus a 33 amino acid peptide of sequence as follows:

G R R R R S V Q W C A V S Q P E A T K C F Q W Q R N M R K V R G P (SEQ ID NO: 2).

When expressed, these peptides may include an N-terminal methionine residue corresponding to a start codon.

Based on the protein sequence, a lactoferricin gene is designed for expression in planta. The codon usage of this synthetic gene is optimized for expression in monocot plants, by using codons for each of the amino acids that are used most frequently in maize

expressed genes. The maize codon usage table has been described by Murray, Lotzer and Eberle, Nucl. Acids Res. 17, 477-498, 1989. Alternatively, the codon usage of the synthetic gene can be optimized following different strategies such as the one described in EP-A-359 472.

The designed sequence for the plant optimized lactoferricin B gene comprises the following coding sequence:

5'-TTC-AAG-TGC-CGC-CGC-TGG-CAG-TGG-CGC-ATG-AAG-AAG-CTG-GGC-GCC-CCC-AGC-ATC-ACC-TGC-GTG-CGC-AGG-GCC-TTC-3' (SEQ ID NO:3)

When a stop codon (TAA) is added to the 3' end, the sequence is as follows:

5'-TTC-AAG-TGC-CGC-CGC-TGG-CAG-TGG-CGC-ATG-AAG-AAG-CTG-GGC-GCC-CCC-AGC-ATC-ACC-TGC-GTG-CGC-AGG-GCC-TTC-TAA-3' (SEQ ID NO:4)

The plant optimized sequence for the lactoferricin H gene is as follows:

5'-GGC-CGC-CGC-CGC-CGC-AGC-GTG-CAG-TGG-TGC-GCC-GTG-AGC-CAG-CCC-GAG-GCC-ACC-AAG-TGC-TTC-CAG-TGG-CAG-CGC-AAC-ATG-CGC-AAG-GTG-CGC-GGC-CCC-3' (SEQ ID NO:5)

Preferably, a methionine start codon and a stop codon (TAG) are added. In addition, the ACC sequence from the Kozak consensus sequence is added to improve the liklihood of efficient translation. This results in the Met-Lactoferricin H gene sequence shown below, with start and stop codons underlined:

5'-ACC-<u>ATG</u>-GGC-CGC-CGC-CGC-CGC-AGC-GTG-CAG-TGG-TGC-GCC-GTG-AGC-CAG-CCC-GAG-GCC-ACC-AAG-TGC-TTC-CAG-TGG-CAG-CGC-AAC-ATG-CGC-AAG-GTG-CGC-GGC-CCC-<u>TAG</u>-3' (SEQ ID NO:6)

A structural coding sequence encoding lactoferricin is expressed in a plant cell under control of a promoter capable of functioning in a plant cell to cause the production of RNA sequences and a 3' non-translated region causing polyadenylation of the transcribed RNA. Examples of promoters capable of functioning in plants or plant cells, i.e., those capable of driving expression of the associated structural genes such as lactoferricin in plant cells, include the cauliflower mosaic virus (CaMV) 19S or 35S promoters and CaMV double promoters; nopaline synthase promoters; pathogenesis-related (PR) protein promoters; small subunit of ribulose bisphosphate carboxylase (ssuRUBISCO) promoters, and the like. Preferred are the rice actin promoter (McElroy *et al., Mol. Gen. Genet. 231*: 150 (1991)), a ubiquitin promoter such as the maize ubiquitin promoter (EP 0 342 926; Taylor *et al., Plant Cell Rep. 12:* 491 (1993)), and the Pr-1 promoter from tobacco, *Arabidopsis*, or maize. Also

preferred are the 35S promoter and an enhanced or double 35S promoter such as that described in Kay *et al., Science 236:* 1299-1302 (1987) and the double 35S promoter cloned into pCGN2113, deposited as ATCC 40587. The promoters themselves may be modified to manipulate promoter strengtn to increase lactoferricin expression, in accordance with art-recognized procedures.

Signal or transit peptides may be fused to the lactoferricin coding sequence in the chimeric DNA constructs of the invention to direct transport of the expressed lactoferricin peptide to the desired site of action. Examples of signal peptides include those natively linked to the plant pathogenesis-related proteins, e.g. PR-1, PR-2, and the like. *See, e.g.*, Payne *et al., Plant Mol. Biol. 11*:89-94 (1988). Examples of transit peptides include the chloroplast transit peptides such as those described in Von Heijne *et al., Plant Mol. Biol. Rep. 9*:104-126 (1991); *Mazur et al., Plant Physiol. 85*: 1110 (1987); Vorst *et al., Gene 65*: 59 (1988), and mitochondrial transit peptides such as those described in Boutry *et al., Nature 328*:340-342 (1987). The relevant disclosures of these publications are incorporated herein by reference in their entirety. For cytoplasmic expression, a methionine start codon is added.

The chimeric DNA construct(s) of the invention may contain multiple copies of a promoter or multiple copies of the lactoferricin structural genes. In addition, the construct(s) may include coding sequences for markers and coding sequences for other peptides such as signal or transit peptides, each in proper reading frame with the other functional elements in the DNA molecule. The preparation of such constructs are within the ordinary level of skill in the art.

Useful markers include peptides providing herbicide, antibiotic or drug resistance, such as, for example, resistance to hygromycin, kanamycin, G418, gentamycin, lincomycin, methotrexate, glyphosate, phosphinothricin, or the like. These markers can be used to select cells transformed with the chimeric DNA constructs of the invention from untransformed cells. Other useful markers are peptidic enzymes which can be easily detected by a visible reaction, for example a color reaction, for example luciferase, ß-glucuronidase, or ß-galactosidase.

Transgenic plants are obtained by

(a) inserting recombinant DNA molecules according to the invention into the genome of a plant cell;

(b) obtaining transformed plant cells; and

(c) regenerating therefrom the transgenic plants which express lactoferricin in an amount effective to reduce damage from disease.

The recombinant DNA molecules can be introduced into the plant cell in a number of art-recognized ways. Those skilled in the art will appreciate that the choice of method might depend on the type of plant, i.e. monocot or dicot, targeted for transformation. Suitable methods of transforming plant cells include microinjection (Crossway *et al., BioTechniques 4*:320-334 (1986)), electroporation (Riggs *et al, Proc. Natl. Acad. Sci. USA 83*:5602-5606 (1986), *Agrobacterium* mediated transformation (Hinchee *et al., Biotechnology 6*:915-921 (1988)), direct gene transfer (Paszkowski *et al., EMBO J. 3*:2717-2722 (1984)), and ballistic particle acceleration using devices available from Agracetus, Inc., Madison, Wisconsin and Dupont, Inc., Wilmington, Delaware (*see, for example,* Sanford *et al.*, U.S. Patent 4,945,050; and McCabe *et al., Biotechnology 6*:923-926 (1988)). Also see, Weissinger *et al., Annual Rev. Genet. 22*:421-477 (1988); Sanford *et al., Particulate Science and Technology 5*:27-37 (1987)(onion); Christou *et al., Plant Physiol. 87*:671-674 (1988)(soybean); McCabe *et al., Bio/Technology 6*:923-926 (1988)(soybean); Datta *et al., Bio/Technology 8*:736-740 (1990)(rice); Klein *et al., Proc. Natl. Acad. Sci. USA, 85*:4305-4309 (1988)(maize); Klein *et al., Bio/Technology 6*:559-563 (1988)(maize); Klein *et al., Plant Physiol. 91*:440-444 (1988)(maize); Fromm *et al., Bio/Technology 8*:833-839 (1990); and Gordon-Kamm *et al., Plant Cell 2*:603-618 (1990)(maize).

The selection of a promoter used in expression cassettes will determine the spatial and temporal expression pattern of the transgene in the transgenic plant. Selected promoters will express transgenes in specific cell types (such as leaf epidermal cells, mesophyll cells, root cortex cells) or in specific tissues or organs (roots, leaves or flowers, for example) and this selection will reflect the desired location of expression of the transgene. Alternatively, the selected promoter may drive expression of the gene under a light-induced or other temporally regulated promoter. A further alternative is that the selected promoter be chemically regulated. This would provide the possibility of inducing expression of the transgene only when desired and caused by treatment with a chemical inducer.

A variety of transcriptional terminators are available for use in expression cassettes. These are responsible for the termination of transcription beyond the transgene and its correct polyadenylation. Appropriate transcriptional terminators and those which are known to function in plants and include the CaMV 35S terminator, the *tml* terminator, the nopaline

synthase terminator, the pea *rbcS* E9 terminator. These can be used in both monocotyledons and dicotyledons.

Numerous sequences have been found to enhance gene expression from within the transcriptional unit and these sequences can be used in conjunction with the genes of this invention to increase their expression in transgenic plants.

Various intron sequences have been shown to enhance expression, particularly in monocotyledonous cells. For example, the introns of the maize *Adh1* gene have been found to significantly enhance the expression of the wild-type gene under its cognate promoter when introduced into maize cells. Intron 1 was found to be particularly effective and enhanced expression in fusion constructs with the chloramphenicol acetyltransferase gene (Callis *et al.*, Genes Develop. 1: 1183-1200 (1987)). In the same experimental system, the intron from the maize *bronze1* gene had a similar effect in enhancing expression (Callis *et al.*, *supra*). Intron sequences have been routinely incorporated into plant transformation vectors, typically within the non-translated leader.

A number of non-translated leader sequences derived from viruses are also known to enhance expression, and these are particularly effective in dicotyledonous cells. Specifically, leader sequences from Tobacco Mosaic Virus (TMV, the "W-sequence"), Maize Chlorotic Mottle Virus (MCMV), and Alfalfa Mosaic Virus (AMV) have been shown to be effective in enhancing expression (*e.g.* Gallie *et al. Nucl. Acids Res. 15*: 8693-8711 (1987); Skuzeski *et al.* Plant Molec. Biol. 15: 65-79 (1990))

Various mechanisms for targeting gene products are known to exist in plants and the sequences controlling the functioning of these mechanisms have been characterized in some detail. For example, the targeting of gene products to the chloroplast is controlled by a signal sequence found at the amino terminal end of various proteins and which is cleaved during chloroplast import yielding the mature protein (*e.g.* Comai *et al.* J. *Biol. Chem. 263*: 15104-15109 (1988)). These signal sequences can be fused to heterologous gene products to effect the import of heterologous products into the chloroplast (van den Broeck *et al. Nature 313*: 358-363 (1985)). DNA encoding for appropriate signal sequences can be isolated from the 5' end of the cDNAs encoding the RUBISCO protein, the CAB protein, the EPSP synthase enzyme, the GS2 protein and many other proteins which are known to be chloroplast localized.

Other gene products are localized to other organelles such as the mitochondrion and the peroxisome (*e.g.* Unger *et al. Plant Molec. Biol. 13*: 411-418 (1989)). The cDNAs

encoding these products can also be manipulated to effect the targeting of heterologous gene products to these organelles. Examples of such sequences are the nuclear-encoded ATPases and specific aspartate amino transferase isoforms for mitochondria. Targeting to cellular protein bodies has been described by Rogers *et al., Proc. Natl. Acad. Sci. USA 82*: 6512-6516 (1985)).

In addition sequences have been characterized which cause the targeting of gene products to other cell compartments. Amino terminal sequences are responsible for targeting to the ER, the apoplast, and extracellular secretion from aleurone cells (Koehler & Ho, *Plant Cell 2:* 769-783 (1990)). Additionally, amino terminal sequences in conjunction with carboxy terminal sequences are responsible for vacuolar targeting of gene products (Shinshi *et al., Plant Molec. Biol. 14*: 357-368 (1990)).

By the fusion of the appropriate targeting sequences described above to transgene sequences of interest it is possible to direct the transgene product to any organelle or cell compartment. For chloroplast targeting, for example, the chloroplast signal sequence from the RUBISCO gene, the CAB gene, the EPSP synthase gene, or the GS2 gene is fused in frame to the amino terminal ATG of the transgene. The signal sequence selected should include the known cleavage site and the fusion constructed should take into account any amino acids after the cleavage site which are required for cleavage. In some cases this requirement may be fulfilled by the addition of a small number of amino acids between the cleavage site and the transgene ATG or alternatively replacement of some amino acids within the transgene sequence. Fusions constructed for chloroplast import can be tested for efficacy of chloroplast uptake by *in vitro* translation of *in vitro* transcribed constructions followed by *in vitro* chloroplast uptake using techniques described by (Bartlett *et al.* In: Edelmann *et al.* (Eds.) Methods in Chloroplast Molecular Biology, Elsevier. pp 1081-1091 (1982); Wasmann *et al. Mol. Gen. Genet. 205*: 446-453 (1986)). These construction techniques are well known in the art and are equally applicable to mitochondria and peroxisomes. The choice of targeting which may be required for expression of the transgenes will depend on the cellular localization of the precursor required as the starting point for a given pathway. This will usually be cytosolic or chloroplastic, although it may in some cases be mitochondrial or peroxisomal. The products of transgene expression will not normally require targeting to the ER, the apoplast or the vacuole.

The above described mechanisms for cellular targeting can be utilized not only in conjunction with their cognate promoters, but also in conjunction with heterologous

promoters so as to effect a specific cell targeting goal under the transcriptional regulation of a promoter which has an expression pattern different to that of the promoter from which the targeting signal derives.

Transformation techniques for dicotyledons are well known in the art and include *Agrobacterium*-based techniques and techniques which do not require *Agrobacterium*. Non-*Agrobacterium* techniques involve the uptake of exogenous genetic material directly by protoplasts or cells. This can be accomplished by PEG or electroporation mediated uptake, particle bombardment-mediated delivery, or microinjection. Examples of these techniques are described by Paszkowski *et al.*, *EMBO J 3:* 2717-2722 (1984), Potrykus *et al.*, *Mol. Gen. Genet. 199:* 169-177 (1985), Reich *et al.*, *Biotechnology 4:* 1001-1004 (1986), and Klein *et al.*, *Nature 327*: 70-73 (1987). In each case the transformed cells are regenerated to whole plants using standard techniques known in the art.

Agrobacterium-mediated transformation is a preferred technique for transformation of dicotyledons because of its high efficiency of transformation and its broad utility with many different species. The many crop species which are routinely transformable by *Agrobacterium* include tobacco, tomato, sunflower, cotton, oilseed rape, potato, soybean, alfalfa and poplar (EP 0 317 511 (cotton); EP 0 249 432 (tomato, to Calgene); WO 87/07299 (*Brassica*, to Calgene); US 4,795,855 (poplar);). *Agrobacterium* transformation typically involves the transfer of the binary vector carrying the foreign DNA of interest (*e.g.* pCIB200 or pCIB2001) to an appropriate *Agrobacterium* strain which may depend of the complement of *vir* genes carried by the host *Agrobacterium* strain either on a co-resident Ti plasmid or chromosomally (*e.g.* strain CIB542 for pCIB200 and pCIB2001 (Uknes *et al.* *Plant Cell 5*: 159-169 (1993)). The transfer of the recombinant binary vector to *Agrobacterium* is accomplished by a triparental mating procedure using *E. coli* carrying the recombinant binary vector, a helper *E. coli* strain which carries a plasmid such as pRK2013 and which is able to mobilize the recombinant binary vector to the target *Agrobacterium* strain. Alternatively, the recombinant binary vector can be transferred to *Agrobacterium* by DNA transformation (Höfgen & Willmitzer, Nucl. Acids Res. 16: 9877(1988)).

Transformation of the target plant species by recombinant *Agrobacterium* usually involves co-cultivation of the *Agrobacterium* with explants from the plant and follows protocols well known in the art. Transformed tissue is regenerated on selectable medium carrying the antibiotic or herbicide resistance marker present between the binary plasmid T-DNA borders.

Transformation of most monocotyledon species has now also become routine. Preferred techniques include direct gene transfer into protoplasts using PEG or electroporation techniques, and particle bombardment into callus tissue. Transformations can be undertaken with a single DNA species or multiple DNA species (*i.e.* co-transformation) and both these techniques are suitable for use with this invention. Co-transformation may have the advantage of avoiding complex vector construction and of generating transgenic plants with unlinked loci for the gene of interest and the selectable marker, enabling the removal of the selectable marker in subsequent generations, should this be regarded desirable. However, a disadvantage of the use of co-transformation is the less than 100% frequency with which separate DNA species are integrated into the genome (Schocher *et al. Biotechnology 4:* 1093-1096 (1986)).

Patent Applications EP 0 292 435, EP 0 392 225 and WO 93/07278 describe techniques for the preparation of callus and protoplasts from an élite inbred line of maize, transformation of protoplasts using PEG or electroporation, and the regeneration of maize plants from transformed protoplasts. Gordon-Kamm *et al.*, *Plant Cell 2:* 603-618 (1990)) and Fromm *et al.*, *Biotechnology 8:* 833-839 (1990)) have published techniques for transformation of A188-derived maize line using particle bombardment. Furthermore, application WO 93/07278 and Koziel *et al.*, *Biotechnology 11:* 194-200 (1993)) describe techniques for the transformation of élite inbred lines of maize by particle bombardment. This technique utilizes immature maize embryos of 1.5-2.5 mm length excised from a maize ear 14-15 days after pollination and a PDS-1000He Biolistics device for bombardment.

Transformation of rice can also be undertaken by direct gene transfer techniques utilizing protoplasts or particle bombardment. Protoplast-mediated transformation has been described for *Japonica*-types and *Indica*-types (Zhang *et al.*, *Plant Cell Rep 7*: 379-384 (1988); Shimamoto *et al. Nature 338*: 274-277 (1989); Datta *et al. Biotechnology 8:* 736-740 (1990)). Both types are also routinely transformable using particle bombardment (Christou *et al. Biotechnology 9:* 957-962 (1991)).

Patent Application EP 0 332 581 describes techniques for the generation, transformation and regeneration of Pooideae protoplasts. These techniques allow the transformation of Pooideae such as *Dactylis* and wheat. Furthermore, wheat transformation has been described by Vasil *et al.*, *Biotechnology 10:* 667-674 (1992)) using particle bombardment into cells of type C long-term regenerable callus, and also by Vasil *et al.*, *Biotechnology 11:* 1553-1558 (1993)) and Weeks *et al.*, *Plant Physiol. 102*: 1077-1084

(1993) using particle bombardment of immature embryos and immature embryo-derived callus. A preferred technique for wheat transformation, however, involves the transformation of wheat by particle bombardment of immature embryos and includes either a high sucrose or a high maltose step prior to gene delivery. Prior to bombardment, any number of embryos (0.75-1 mm in length) are plated onto MS medium with 3% sucrose (Murashige & Skoog, *Physiologia Plantarum 15*: 473-497 (1962)) and 3 mg/l 2,4-D for induction of somatic embryos which is allowed to proceed in the dark. On the chosen day of bombardment, embryos are removed from the induction medium and placed onto the osmoticum (*i.e.* induction medium with sucrose or maltose added at the desired concentration, typically 15%). The embryos are allowed to plasmolyze for 2-3 h and are then bombarded. Twenty embryos per target plate is typical, although not critical. An appropriate gene-carrying plasmid (such as pCIB3064 or pSG35) is precipitated onto micrometer size gold particles using standard procedures. Each plate of embryos is shot with the DuPont Biolistics® helium device using a burst pressure of ~1000 psi using a standard 80 mesh screen. After bombardment, the embryos are placed back into the dark to recover for about 24 h (still on osmoticum). After 24 hrs, the embryos are removed from the osmoticum and placed back onto induction medium where they stay for about a month before regeneration. Approximately one month later the embryo explants with developing embryogenic callus are transferred to regeneration medium (MS + 1 mg/liter NAA, 5 mg/liter GA), further containing the appropriate selection agent (10 mg/l basta in the case of pCIB3064 and 2 mg/l methotrexate in the case of pSOG35). After approximately one month, developed shoots are transferred to larger sterile containers known as "GA7s" which contained half-strength MS, 2% sucrose, and the same concentration of selection agent.

Lactoferricin when expressed in plants controls or inhibits a broad spectrum of plant pathogens, including viral, bacterial and fungal plant pathogens. Commercially important plant diseases suitable for control by expression of lactoferricin in plants include the following:

Corn pathogens

Fungal: *Gibberella zeae*
Aspergillus flavus
A. parasiticus
Fusarium moniliforme
Diplodia maydis
Colletotrichum graminicola
Cephalosporium acremonium
Macrophomina phaseolina
Cercospora zeae-maydis
Exserohilum turcicum
Bipolaris maydis
Kabatiella zeae
Puccinia sorghi
Puccinia polysora
Sphacelotheca reiliana
Ustilago maydis
Colletotrichum graminicola
Helminthosporium carbonum
Peronosclerospora sorghi
Sclerophthora rayssiae
Peronosclerospora sacchari
Peronoscler. philippinensis
Peronosclerospora maydis
Peronosclerospora spontanea
Peronosclerospora heteropogoni
Sclerospora graminicola

Bacterial: *Erwinia stewartii*
Corynebacterium nebraskense

Viral: Maize dwarf mosaic virus
Maize rough dwarf (Maize rio cuarto) virus
Maize streak virus
Maize chlorotic dwarf virus

Maize chlorotic mottle virus

Barley yellow dwarf virus

Corn lethal necrosis virus

High plains virus

Maize stripe virus

Sweet corn pathogens:

A. Bacterial Diseases

Erwinia stewartii

Pseudomonas avenae

Erwinia chrysanthemi

B. Fungal Diseases

Bipolaris maydis

Exserohilum turcicum

Colletotrichum graminicola

Phyllosticta maydis

Kabatiella zeae

Cercospora zeae-maydis

Sclerophthora spp.

Peronosclerospora spp.

Ustilago maydis

Sphacelotheca reiliana

Puccinia sorghi

Puccinia polysora

Physopella zeae

Fusarium moniliforme

Pythium spp.

Penicillium oxalicum

Fusarium spp.

Diplodia maydis

Gibberella zeae

C. Viral Diseases

Sugarcane Mosaic

Maize Dwarf Mosaic

Maize Chlorotic Dwarf

High Plains Virus

Maize Chlorotic Mottle

Wheat Streak Mosaic

Barley Yellow Dwarf

Wheat pathogens

Fungal pathogens:

Puccinia graminis f.sp. tritici

Puccinia recondita f.sp. tritici

Puccinia striiformis

Tilletia tritici

Tilletia controversa

Tilletia indica

Ustilago tritici

Urocystis tritici

Gaeumannomyces graminis

Pythium spp.

Fusarium culmorum

Fusarium graminaerum

Fusarium avenaceum

Drechslere tritici-repentis

Rhizoctonia spp.

Colletotrichum graminicola

Helminthosporium spp.

Microdochium nivale

Pseudocercosporella herpotrichoides

Erysiphe graminis f.sp. tritici

Sclerophthora macrospora

Septoria tritici

Septoria nodorum (Stagonospora nodorum)

Bipolaris sorokiniana

Fusarium roseum

Cephalosporium gramineum

Cochliobolus sativus

Claviceps purpurea

Bacterial pathogens:

Pseudomonas syringae pv. atrofaciens

Pseudomonas syringae pv. syringae

Xanthomonas campestris pv. translucens

Viral pathogens:

Barley Yellow Dwarf virus

Soilborne Wheat Mosaic virus

Wheat Spindle Streak Mosaic virus

Wheat Yellow Mosaic virus

Soybean pathogens

1. Fungal

Phytophthora megasperma var. sojae

Colletotrichum truncatum

Septoria glycines

Cercospora kikuchii

Cercospora sojina

Peronospora manshurica

Microsphaera diffusa

Rhizoctonia solani

Phakopsora pachyrhizi

Corynespora cassicola

Calonectria crotalariae

Fusarium solani

Fusarium oxysporum

Macrophomina phaseolina

Diaporthe phaseolorum var caulivora & meridionalis

Diaporthe phaseolorum var. sojae

Phialophora gregata

Pythium spp

Sclerotinia sclerotiorum

Sclerotiun rolfsii

Thielaviopsis basicola

2. Bacterial

Pseudomonas syringae pv. glycinea

Xanthomonas campestris pv. phaseoli

3. Viral

Soybean mosaic

Bean pod mottle

Bud blight

Peanut mottle

Cowpea Chlorotic Mottle

Yellow Mosaic

Pea pathogens

I. Seed & Seedling Diseases

Pythium ultimum

Rhizoctonia solani

II. Diseases Caused by Bacteria

Pseudomonas syringae pv. pisi

Pseudomonas syringae pv. syringae

III. Foliar Diseases Caused by Fungi

Ascochyta pisi

Mycosphaerella pinodes

Ascochyta pinodella

Sclerotinia sclerotiorum

Peronospora pisi

Erysiphe pisi

Colletotrichum pisi

Alternaria alternata

Botrytis cinerea

IV. Root Diseases Caused by Fungi

Fusarium oxysporum f. sp. pisi

Aphanomyces euteiches f. sp. pisi

Pythium ultimum

Fusarium solani f. sp. pisi

Thielaviopsis basicola

V. Diseases Caused by Viruses

Pea Enation Mosaic

Bean (Pea) Leaf Roll

Pea Seedborne Mosaic

Pea Streak

Pea Mosaic

Cucumber Mosaic

Pea Early Browning Virus

Red Clover Vein Mosaic

Bean pathogens

I. Root Diseases

Aphanomyces euteiches f. sp. phaseoli

Pythium ultimum

Rhizoctonia solani

Fusarium solani f. sp. phaseoli

Thielaviopsis basicola

Sclerotium rolfsii

II. Fungal Diseases of Aerial Parts

Colletotrichum lindemuthianum

Uromyces appendiculatus

Sclerotinia sclerotiorum

Fusarium oxysporum f. sp. phaseoli

Phoma exigua var exigua

Cercospora canescens

Chaetoseptoria wellmanii

Phytophthora nicotianae

Erysiphe polygoni

Pythium ultimum & debaryanum

Alternaria alternata, various species

Phaeoisariopsis griseola

Macrophomina phaseolina

Botrytis cinerea

Rhizoctonia solani

III. Diseases Caused by Bacteria

Pseudomonas syringae pv. phaseolicola

Pseudomonas syringae pv. syringae

Xanthomonas phaseoli

IV. Diseases Caused by Nematodes

Meloidogyne incognita

Pratylenchus species

V. Diseases Caused by Viruses

Bean Common Mosaic

Bean Golden Mosaic

Curly Top

Bean Curly Dwarf Mosaic

Bean Pod Mottle

Clover Yellow Vein

Red Node

Cucumber Mosaic

Peanut Stunt

Sugar beet pathogens

1. Fungal:

Cercospora beticola	leaf spot disease
Aphanomyces cochlioides	
Rhizoctonia solani	damping off, root rot
Erysiphe betae	powdery mildew
Rhizoctonia violacea (Helicobasidium purpureum)	
Ramularia beticola	
Pythium spp.	
Phoma betae	
Uromyces betae	
Peronospora farinosa	
Alternaria tenuis	
Fusarium oxysporium	
Verticillium dahliae	
Sclerotium rolfsii	
Erysiphe polygoni	powdery mildew
Polymyxa betae	

2. Bacterial:

Pseudomonas syringae	
Erwinia carotovora	Erwinia root rot

3. Viral:

Rhizomania Beet necrotic yellow vein virus (BNYVV)

Beet mild yellowing virus (BMYV)

Beet yellows virus (BYV)

Beet curly top virus

Beet mosaic virus

The invention provides plants and seed, which when germinated and cultivated, express lactoferricin. Preferably, lactoferricin is expressed from recombinant DNA stably integrated into the genome. The transgenic plants or seed are either monocotyledoneous or dicotyledoneous and preferably selected from the group consisting of maize, rice, wheat, barley, sorghum, rye, oats, millet, cotton, soybeans, peas, beans, sunflower, grasses and oil seed rape. The trangenic seed can be packaged into a bag preferably containing lable instructions for the use thereof to control plant pathogens.

The genetic properties engineered into the transgenic seeds and plants described above are passed on by sexual reproduction or vegetative growth and can thus be maintained and propagated in progeny plants. Generally said maintenance and propagation make use of known agricultural methods developed to fit specific purposes such as tilling, sowing or harvesting. Specialized processes such as hydroponics or greenhouse technologies can also be applied. As the growing crop is vulnerable to attack and damages caused by insects or infections as well as to competition by weed plants, measures are undertaken to control weeds, plant diseases, insects, nematodes, and other adverse conditions to improve yield. These include mechanical measures such a tillage of the soil or removal of weeds and infected plants, as well as the application of agrochemicals such as herbicides, fungicides, gametocides, nematicides, growth regulants, ripening agents and insecticides.

Use of the advantageous genetic properties of the transgenic plants and seeds according to the invention can further be made in plant breeding which aims at the development of plants with improved properties such as tolerance of pests, herbicides, or stress, improved nutritional value, increased yield, or improved structure causing less loss from lodging or shattering. The various breeding steps are characterized by well-defined human intervention such as selecting the lines to be crossed, directing pollination of the parental lines, or selecting appropriate progeny plants. Depending on the desired properties different breeding measures are taken. The relevant techniques are well known in the art and include but are not limited to hybridization, inbreeding, backcross breeding, multiline breeding, variety blend, interspecific hybridization, aneuploid techniques, etc. Hybridization techniques also include the sterilization of plants to yield male or female sterile plants by mechanical, chemical or biochemical means. Cross pollination of a male sterile plant with pollen of a different line assures that the genome of the male sterile but female fertile plant

will uniformly obtain properties of both parental lines. Thus, the transgenic seeds and plants according to the invention can be used for the breeding of improved plant lines which for example increase the effectiveness of conventional methods such as herbicide or pestidice treatment or allow to dispense with said methods due to their modified genetic properties. Alternatively new crops with improved stress tolerance can be obtained which, due to their optimized genetic "equipment", yield harvested product of better quality than products which were not able to tolerate comparable adverse developmental conditions.

In seeds production germination quality and uniformity of seeds are essential product characteristics, whereas germination quality and uniformity of seeds harvested and sold by the farmer is not important. As it is difficult to keep a crop free from other crop and weed seeds, to control seedborne diseases, and to produce seed with good germination, fairly extensive and well-defined seed production practices have been developed by seed producers, who are experienced in the art of growing, conditioning and marketing of pure seed. Thus, it is common practice for the farmer to buy certified seed meeting specific quality standards instead of using seed harvested from his own crop. Propagation material to be used as seeds is customarily treated with a protectant coating comprising herbicides, insecticides, fungicides, bactericides, nematicides, molluscicides or mixtures thereof. Customarily used protectant coatings comprise compounds such as captan, carboxin, thiram (TMTD®), methalaxyl (Apron®), and pirimiphos-methyl (Actellic®). If desired these compounds are formulated together with further carriers, surfactants or application-promoting adjuvants customarily employed in the art of formulation to provide protection against damage caused by bacterial, fungal or animal pests. The protectant coatings may be applied by impregnating propagation material with a liquid formulation or by coating with a combined wet or dry formulation. Other methods of application are also possible such as treatment directed at the buds or the fruit.

It is a further aspect of the present invention to provide new agricultural methods such as the methods examplified above which are characterized by the use of transgenic plants, transgenic plant material, or transgenic seed according to the present invention to provide control against plant diseases

To breed progeny from plants transformed according to the method of the present invention, a method such as that which follows may be used: plants produced as described

in the examples set forth below are grown in pots in a greenhouse or in soil, as is known in the art, and permitted to flower. Pollen is obtained and used to pollinate the same plant, sibling plants, or any desirable plant. Similarly, the transformed plant may be pollinated by pollen obtained from the same plant, sibling plants, or any desirable maize plant. Transformed progeny obtained by this method may be distinguished from non-transformed progeny by the presence of the introduced gene(s) and/or accompanying DNA (genotype), or the phenotype conferred. The transformed progeny may similarly be selfed or crossed to other plants, as is normally done with any plant carrying a desirable trait. Similarly other transformed plants produced by this method may be selfed or crossed as is known in the art in order to produce progeny with desired characteristics.

The invention thus provides, in a first embodiment,

1. A recombinant DNA molecule comprising in operative sequence:

(a) a promoter which functions in plant cells to cause the production of an RNA sequence, e.g., a promoter as described above, for example the maize ubiquitin promoter;

(b) a structural coding sequence that encodes for production of lactoferricin, e.g., lactoferricin B or lactoferricin H, e.g., a peptide of SEQ ID NO:1 or 2 optionally having an N-terminal methionine; preferably a plant optimized coding sequence, e.g., of SEQ ID NO:3, 4, 5, or 6; and

(c) a 3' non-translated region which functions in plant cells to cause the addition of polyadenylate nucleotides to the 3' end of the RNA sequence, e.g., a transcriptional terminator as described above, for example, the nopaline synthase transcriptional terminator from *Agrobacterium tumifaciens*;

and optionally further comprising one or more enhancers or targeting sequences, as described herein.

In a second embodiment, the invention provides

2. A method of producing transgenic disease resistant plants, e.g., plants as described above, for example maize or wheat plants capable of expressing lactoferricin, comprising the steps of:

(a) inserting into the genome of a plant cell, a recombinant DNA molecule as described above, e.g., comprising (i) a promoter which functions in plant cells to cause the

production of an RNA sequence; (ii) a structural coding sequence that encodes for the production of lactoferricin; and (iii) a 3' non-translated region which functions in plant cells to cause the addition of polyadenylate nucleotides to the 3' end of the RNA sequence;

(b) obtaining transformed plant cells, and

(c) regenerating from the transformed plant cells genetically transformed plants which express lactoferricin in an amount effective to reduce damage from infection by a pathogen.

In a third embodiment, the invention provides

3. A plant (e.g., a maize, wheat or sugar beet plant) comprised of cells which express lactoferricin,

for example, a plant having stably integrated in its genome recombinant DNA comprising in operative sequence:

(a) a promoter which functions in plant cells to cause the production of an RNA sequence;

(b) a structural coding sequence that encodes for production of lactoferricin; and

(c) a 3' non-translated region which functions in plant cells to cause the addition of polyadenylate nucleotides to the 3' end of the RNA sequence;

e.g., wherein the plant is developmentally and morphologically normal.

In a fourth embodiment, the invention provides

4. Seed which when germinated and cultivated will produce a plant comprised of cells which express lactoferricin, e.g., a plant as described above, e.g., wherein the seed is optionally coated (e.g., with polymers, fungicides, insecticides, dyes or other seed coatings) and/or packaged, e.g., in a bag or other container for sale or transport.

In a fifth embodiment, the invention provides

5. Use of lactoferricin to control plant pathogens, e.g., plant bacterial, fungal or viral pathogens, e.g., as described above;

and a method of controlling plant pathogens, e.g., as described above, comprising exposing the pathogens to a plant which expresses lactoferricin.

The following description further exemplifies the methods and compositions of this invention. However, it will be understood that that other methods, known by those of ordinary skill in the art to be equivalent, can also be employed.

Examples

Example 1: Antimicrobial activity of Lactoferricin B on plant pathogenic fungi and other micro-organisms.

a. Inhibition of Spore Germination

The activity of lactoferricin B was tested in spore germination inhibition assays using *Diplodia maydis* and *Colletotrichum graminicola* spores. The fungal strains were obtained from Loral Castor, CIBA-GEIGY, Illinois. The lactoferricin B peptide and lactoferrin were obtained from Morinaga Milk Industry Co, LTD, Tokyo, Japan.

Fungal spores were harvested from sporulating cultures grown on potato dextrose agar (PDA, Difco) plates. D. maydis spores were used fresh, or stored at -80°C in 25% glycerol. C. graminicola spores were used fresh.

For the assay, 10 ml molten medium (1.5% agar, containing 8% carrot extract) was cooled to approximately 50°C. Ten microliter streptomycin (250 mg/ml) was added, followed by 100 microliter of either *D. maydis* or *C. graminicola* spores, at concentrations of approximately 10^6/ml (*D. maydis*) and 10^7/ml (*C. graminicola*). The solutions were mixed by gentle swirling and poured into sterile petridishes. After the medium solidified, sterile 1/4 inch diameter filter discs were placed on top of the medium and test solutions were pipetted onto these disks. Test solutions were lactoferrin (10 microgram) lactoferricin B (10 microgram), purothionin (the positive control, at 0, 5, 10 and 20 microgram; purchased from Calbiochem) and buffer (20 mM Tris pH7.5).

After incubation at roomtemperature for 2-5 days, fungal growth was clearly visible on the plates, except around the filterdiscs containing purothionin and lactoferricin B. The sizes of the inhibition zones are shown in 1. In the D. maydis experiment, the filterdisk with 10 microgram lactoferricin B showed a zone of inhibition of 4.95 mm, whereas 20 microgram purothionin showed an inhibition zone of 4.5 mm. In the C. graminicola experiment, lactoferricin B produced a zone of inhibition of 1.7 mm, whereas purothionin produced an inhibition zone of 2.1 mm. Lactoferrin did not cause inhibition of fungal germination and growth.

Erwinia stewartii was also tested in this system and it was shown that lactoferricin B also inhibits growth of this bacterium.

Table 1: Inhibition of spore germination by lactoferricin B

Fungus	zone of inhibition		
	Buffer	Purothionin 20µg	Lactoferricin B 10µg
D. maydis	0 mm	4.5 mm	4.95 mm
C. graminicola	0 mm	2.1 mm	1.7 mm

b. Inhibition of mycelial growth

A second method used to evaluate antifungal activity involved measuring inhibition of mycelial growth. In this assay, wells were punched with the broad end of a Pasteur pipette at the perimeter of 1.5% agarose plates containing 4% carrot extract. The bottom of the wells was sealed with a drop of the molten agarose solution. Individual fungal plugs were taken from a master plate of either Bipolaris maydis, Fusarium monoliforme, Gibberella zeae, Pythium aphanidermatum, or Rhizoctonia solani and centrally placed on each test plate. Test solutions placed in the wells (40 microliter) were lactoferricin B (100 microgram), lactoferrin (100 microgram) or buffer (20 mM Tris pH7.5). After incubation at roomtemperature for the time required for the fungi to have grown from the inoculated plug to the wells, inhibition of fungal growth was scored. All fungi showed inhibition of growth near the wells containing lactoferricin B, but not near the lactoferrin or buffer control well. The results are shown in table 2.

Table 2: Inhibition of fungal growth by lactoferricin B.

Fungus	growth inhibition (mm)	
	Buffer	Lactoferricin B (100 µg)
Bipolaris maydis	0	8
Fusarium monoliforme	0	9.7
Gibberella zeae	0	9.8
Pythium aphanidermatum	0	20.9
Rhizoctonia solani	0	1.6

c. Minimum Inhibitory Concentration in liquid culture

The activity of lactoferricin B and 2 derivatives on Fusarium culmorum and Septoria nodorum was tested in liquid inhibition assays. The peptides were lactoferricin B (as described above, Met-lactoferricin B (lactoferricin B with an additional N-terminal methionine), and Gln-lactoferricin B (lactoferricin B with an additional N-terminal glutamine). All peptides were purchased from the W.M. Keck Biotechnology Resource Center, New Haven, CT. Peptides were disolved in double distilled sterile water at 1.5 mg/ml.

Fungal spores were harvested from sporulating cultures grown on potato dextrose agar (PDA, Difco) plates as descibed above and diluted to 15,000/ml in 1/2 strength PDA. To 50 microliter spores, 25 microliter peptide of a dilution series was added, all in 96 well plates. After mixing the wells, the OD at 590 nm was measured (a measure for the growth of the fungi). The plates were next incubated at 28°C and the OD at 590 nm was measured at intervals over the next 2 days. The minimum concentration of the lactoferricin B peptides that totally inhibited growth was expressed as the Minimum Inhibitory Concentration (MIC). These values are shown in Table 3.

Pichia pastoris was grown on YPD plates and Pseudomonas syringae BL882 and E. coli DH5α in L broth. A small amount of P. pastoris cells was resuspended in YPD, the cell concentration was counted and adjusted to 30,000 cells/ml with YPD. The OD at 600 nm was measured of cultures of DH5α and BL882. Cell concentrations were adjusted to 30,000, cells/ml in L broth, assuming that an OD=1 at 600nm for DH5α corresponded to 109 cells/ml and $5x10^8$ cells/ml for BL882.

To 80 microliter cells, 20 microliter of a dilution series of lactoferricin B peptides was added, all in 96 well plates. The OD at 590 nm of the plates was next read and next the plates were incubated at 28°C. The OD at 590 was read regularly over the next 2 days. The minimum concentration of the lactoferricin B peptides that totally inhibited growth was expressed as the Minimum Inhibitory Concentration (MIC). These values are shown in Table 3.

Table 3: Minimum Inhibitory Concentrations of Lactoferricin B

Organism	Peptide		
	lactoferricin B	Met-lactoferricin B	Gln-lactoferricin B
F. culmorum	20	20	20
S. nodorum	100	100	100
C. graminicola	100	100	100
P. syringae BL882	20	20	20
E. coli DH5α	100	100	100
Pichia pastoris	300	300	300

Example 2: Stability of the Gln-lactoferricin B peptide in plant extracellular fluid

The lactoferricin B peptide is expressed in plants using control sequences that direct the peptide to several different subcellular locations (described in detail in Examples 3-6, below). For extracellular expression, the coding sequence of lactoferricin B is fused to the leader sequence of the maize PR-1 gene, which causes secretion of the peptide into the extracellular space. The leader sequence used for this purpose includes the glutamine residue at the start of the mature, secreted PR-1 protein and thus results in secretion of a lactoferricin B peptide with an additional N-terminal glutamine residue (Gln-lactoferricin B). A synthetic Gln-lactoferricin B peptide was tested for its stability in extracellular wash fluid of wheat, corn and tobacco.

Extracellular wash fluid (ECF) was obtained by infiltrating leaf tissue with sterile, double distilled water, which was recovered by centrifugation of the infiltrated tissue. For wheat, a leaf of a UC703 plant was used, and for corn, a leaf of a 6N615 plant. These leaves were cut into small pieces that were placed in the barrel of a 20 ml syringe, which contained cottonwool in the bottom. The cuttings were washed with water to remove cellular exudate from the cut surfaces. Next, water was added and the plunger placed on the syringe barrel. The syringe was placed upside down in a lyophilizer vessel, which was then connected to the lyophilizer. After applying a gentle vacuum for 30-40 seconds, the vacuum was released. This was repeated once. The leaf pieces were next placed in the barrel of a 10 ml syringe, which was placed in a Centrifuge tube. This was spun at 1000 to 2000 rpm in a clinical centrifuge, to recover the extracellular wash fluid, which was next frozen.

For tobacco (cultivar Xanthi.nc), a leaf was injected with water, using a 10 ml syringe. The leaf was next removed from the plant, blotted dry, rolled up and cut into short cylinders

that were placed in a 10 ml syringe barrel. The syringe was placed in a centrifuge tube and next spun in a clincal centrifuge at 1000 rpm. The wash fluid that collected in the tube was collected and frozen.

Aliquots of the ECF were run on a 10% Tricine gel according to the manufacturers instructions (Novex). An estimate of the relative protein concentration was made for each plant ECF. This was used to make an estimate of the amount of ECF needed from each collected fluid to have approximately equal amounts of total ECF protein in the subsequent incubation. Thus, 18.5 microliter ECF from wheat, 31 microliter corn ECF and 60 microliter tobacco ECF were each incubated with 11.25 microgram Gln-lactoferricin B peptide, in a total volume of 75 microliter, containing 10 mM Tris pH7.5, 50 mM NaCl, 2.5 mM $MgCl_2$ and 2.5 mM $CaCl_2$. The reactions were incubated at 37°C and 10 microliter aliquots were taken at 0, 15, 30, 60, 90, 120 and 180 minutes. These samples were immediately frozen.

The samples were analyzed for the presence of Gln-lactoferricin B by gel electrophoresis, after heat inactivation and alkylation. Following a 10 minute incubation at 100°C, the samples were cooled and quick spun in an eppendorf centrifuge. The alkylation of each sample was accomplished by first reducing the samples with 2 microliter 1 mM DTT in an N_2 atmosphere for 10 minutes at 100°C. Next, iodoacetamide was added (2 microliter of 10 mM) and 1.4 microliter 1M Tris (pH 8.5), to make the solution more basic. After flushing the tubes with N_2, the samples were incubated in the dark at 50°C for 50 minutes. Control peptide samples (Gln-lactoferricin B peptide without buffer or ECF) were alkylated in a similar manner. The samples were stored frozen until analyzed by gel electrophoresis.

To each sample, an equal amount of 2xTricine/SDS sample buffer was added (Novex) after which they were incubated at 100°C for 5 minutes. The samples were next run on a 10-20% Tricine/SDS gel (Novex), according to the manufacturer's instructions. The gel was stained with Coomassie (Pharmacia) and destained in order to visualize the peptide bands.

The Gln-lactoferricin B peptide was quite stable in wheat ECF. After a 3 hour incubation, substantial amounts of peptide are still present, indicating that the degradation of the peptide is relatively slow in this ECF. In corn and tobacco ECF, Gln-lactoferricin B peptide is also still present after the 3 hour incubation, albeit at decreased levels.

The results indicate that the Gln-lactoferricin B peptide is only slowly degraded in the ECF of these 3 plant species. Degradation of the peptide in ECF takes over 3 hours under these conditions.

Example 3: Construction of a gene for cytoplasmic expression of lactoferricin B in plants

The coding and noncoding DNA sequences for the cytoplasmicaliy expressed synthetic lactoferricin B gene were obtained by synthesis of 2 partly complementary oligonucleotides. The oligonucleotides encoded (from 5' to 3' on the coding strand) a BamHI restriction enzyme end, a Kozak consensus site, including a methionine start codon, 75 base pairs encoding the amino acid sequence of the lactoferricin B peptide, a stop codon, and a NotI restriction enzyme end.
The nucleotide sequences of the oligos were:

Oligo 1 (SEQ ID NO:7):
5'-GATCCACCATGTTCAAGTGCCGCCGCTGGCAGTGGCGCATGAAGAAGCTGGGCGCCCCCAGC-
ATCACCTGCGTGCGCAGGGCCTTCTAAGC-3'

Oligo 2 (SEQ ID NO:8):
5'-GGCCGCTTAGAAGGCCCTGCGCACGCAGGTGATGCTGGGGGCGCCCAGCTTCTTCATGCGCC-
ACTGCCAGCGGCGGCACTTGAACATGGTG-3'

These synthetic oligonucleotides were purchased and electrophoresed on a 10% urea/polyacrylamide gel and the bands containing the full length oligonucleotides were excised. The oligonucleotides were eluted, annealed and used for PCR amplification using oligos 1-1 and 2-1.

Oligo 1-1 (SEQ ID NO:9):
5'-AGTAGGATCCACCATGTTCAAGTGCC-3'

Oligo 2-1 (SEQ ID NO:10):
5'-TACTGCGGCCGCTTAGAAGGCCCTGCGC-3'

The PCR product was ligated into pCRII, clones containing the insert were identified and inserts were sequenced to confirm the absence of introduced mutations. The BamHI-NotI insert of a clone with the correct sequence was cloned into BamHI and NotI predigested Bluescript. A 2 kb HindIII-BamHI fragment containing the ubiquitin promoter (Toki, et al. Plant Physiol. 100: 1503) was cloned into the HindIII - BamHI sites of this clone. The nos terminator was ligated into the NotI-SacI sites to generate pCIB7703 (shown in figure 1), a plasmid with the ubiquitin-lactoferricin B-nos gene cassette. A DNA fragment

containing ubi-SynPAT-nos was cloned into the HindIII site of this plasmid. The resulting plasmid, pCIB7704 (shown in figure 2), contained the ubi-SynPAT-nos and ubi-lacto-nos gene cassettes. This plasmid contains the SynPAT gene for selection in plants and is useful for transformation of plants with the lactoferricin B gene under control of the maize ubiquitin promoter.

Plasmid pCIB7704 is cleaved with KpnI and SacI to release the fragment containing the ubi-SynPAT-nos -- ubi-lacto-nos gene fusions. This fragment is cloned into KpnI, SacI digested pCIB6848 to form pCIB7705 (shown in figure 3). Plasmid pCIB7705 contains the NPTII (kanamycin resistance) gene for selection in bacteria and the SynPAT gene for selection in plants, and is useful for transformation of plants with the lactoferricin B gene under control of the maize ubiquitin promoter.

The KpnI-SacI fragment from pCIB7703, containing the ubi-lacto-nos construct is cloned into plasmid pCIB6848, to generate pCIB7706 (shown in figure 4). This places the ubi-lacto-nos gene construction in a kanamycin resistance gene containing plasmid.

Example 4: Construction of a synthetic gene for extracellular expression of lactoferricin B in plants

For extracellular expression of lactoferricin B, a leader sequence was placed at the 5' end of the lactoferricin B gene. The leader sequence was derived from a maize PR1 cDNA (PR-1mz) that was cloned from a maize cDNA library (using a barley PR-1 gene probe) and sequenced (patent publication WO 95/19443). Synthetic oligonucleotides and PCR amplification are used to make this gene construct, in 2 steps. Oligonucleotide 3 was identical to the PR-1 leader and the start of the lactoferricin B coding sequence, with the exception of a single silent nucleotide change (to prevent potential secondary structure problems). Oligo 4 was complementary to the lactoferricin B coding sequence (the design of which is described in the detailed description), and the 3' end of the PR-1 leader sequence. Oligo 3-1 was identical to the 5' end of the PR-1 coding sequence.

The nucleotide sequences of the synthetic oligonucleotides were:

Oligo 3 (SEQ ID NO:11):

5'-GATCCACCATGGCACCGAGGCTAGCGTGCCTCCTAGCTCTGGCCATGGCAGCCATCGTCGTGG-
CGCCATGCACGGCCTTCAAGTGCCG-3'

Oligo 4 (SEQ ID NO:12):

5'-GGCCGCTTAGAAGGCCCTGCGCACGCAGGTGATGCTGGGGGCGCCCAGCTTCTTCATGCGCCA-
CTGCCAGCGGCGGCACTTGAAGGCC-3'

Oligo 3-1 (SEQ ID NO:13):

5'-AGTAGGATCCACCATGGCACCGAGGCTAG-3'

Oligo 5 (SEQ ID NO:14):

5'-AGTACCATCGTCGTGGCGCCATGCACGGCCCAGTTCAAG-3'

First, the maize PR1 leader sequence was PCR amplified using oligos 3-1 and 4, with the maize PR-1 cDNA clone as template. This generated a PCR fragment containing an intact BamHI restriction site, the PR-1 leader, lactoferricin B and part of the NotI restriction site. The lactoferricin B template was used in a PCR reaction, using as primers oligo 2-1 (described in Example 3 above) and oligo 3. These 2 PCR fragments were next mixed, denatured at 94°C and extended with Taq DNA polymerase for 5 minutes at 72°C. Next, this reaction was PCR amplified using oligos 2-1 and 3-1 for 10 cycles.

The PCR fragment was cloned into PCRII. Clones containing the insert were identified and the inserts sequenced to confirm the absence of introduced mutations. A plasmid, #10, containing an insert with one mutation in the leader sequence was isolated (GCC changed to a GAC at the twelfth amino acid). In a repeat of the above experiment, a plasmid, #11, with one mutation in the lactoferricin B gene sequence was isolated (GCC changed to an ACC at the fifteenth amino acid). These two plasmids, each containing a different mutation, were used to produce a corrected sequence. The BamHI-NotI inserts were cloned into pBluescript as BamHI-NotI inserts. A BstXI fragment containing the mutation was removed from the Bluescript plasmid containing insert #11 and replaced with the corresponding BstXI fragment from the Bluescript plasmid containing insert#10. A 2 kb HindIII-BamHI fragment containing the ubiquitin promoter (Toki, et al. Plant Physiol. 100: 1503) was cloned into the HindIII - BamHI sites of this clone and the nos terminator was ligated into the NotI-SacI sites. The resulting plasmid was called p#10/11.

This corrected plasmid was next used as a template in a PCR reaction, using as primers oligo 5 and oligo 2-1. Oligo 5 added one amino acid, a glutamine, before the first amino acid of lactoferricin B. This was done to generate an authentic leader cleavage site at the junction of the PR1 leader and the lactoferricin B sequence. Removal of the PR1 leader in

plant cells is thus expected to generate a lactoferricin B peptide with an added N-terminal glutamine.

The resulting PCR fragments were cloned into pCRII and clones were sequenced. A BstXI-NotI insert with the correct sequence (containing the additional glutamine) was isolated and next cloned into BstXI, NotI digested p#10/11 to generate pCIB7707. This placed the maize PR1 leader-lactoferricin B coding sequence under control of the ubiquitin promoter and the nos terminator (ubi-PR1-lactoferricin B-nos). A DNA fragment containing ubi-SynPAT-nos was cloned into the HindIII site of this plasmid, resulting in plasmid pCIB7708, which contains the SynPat and PR1-lactoferricin B gene cassettes.

This plasmid is cleaved with KpnI and SacI to release the insert containing the ubi-SynPAT-nos -- ubi-PR1-lacto-nos gene fusions. This fragment is cloned into KpnI, SacI digested pCIB6848 to form pCIB7709. This plasmid contains the NPTII (kanamycin resistance) gene for selection in bacteria and the SynPAT gene for selection in plants, and is useful for transformation of plants with the lactoferricin B gene under control of the maize ubiquitin promoter.

The KpnI-SacI fragment from pCIB7707, containing the ubi-lacto-nos cassette is cloned into plasmid pCIB6848, to generate pCIB7710. This places the ubi-lacto-nos gene construction in a kanamycin resistance gene containing plasmid.

Example 5: Construction of a gene for vacuolar expression of lactoferricin B in plants

For vacuolar expression of lactoferricin B, a sequence for vacuolar targeting was added to the 3' end of the PR1 leader-lactoferricin B gene cassette described in Example 4 (Construction of a synthetic gene for extracellular expression of lactoferricin B in plants) above. This additional 18 base pair sequence encoded a C-terminal Val-Phe-Ala-Glu-Ala-Ile vacuole targeting sequence (Dombrowski et al., Plant Cell 5, 587-596, 1993) and was followed by a stop codon. In addition, restriction ends were incorporated at the 5' and 3' ends of the synthetic gene, to facilitate cloning.

Two oligonucleotides were used to construct this synthetic gene. The sequence of oligo 3-1 is described in Example 4 above. The sequence of oligo 6 contains the complement of the 3' end of the lactoferricin B coding sequence, the codons for the vacuole targetting sequence, a stopcodon and a NotI restriction enzyme site. Its sequence is listed below.

Oligo 6 (SEQ ID NO:15):

5'-AGTAGCGGCCGC-TTA-GATGGCCTCGGCGAACAC-GAAGGCCCTGCGCACGCA-3'

Oligo 3-1 and oligo 6 were used to PCR amplify the PR1 leader-lactoferricin B gene construct in plasmid pCIB7707, described in Example 4 above. The PCR fragment was cloned into pCRII and clones with insert were selected. The inserts were sequenced and a clone with the correct sequence was used to excise the BamHI-NotI fragment. This fragment was cloned into Bluescript, and a 2 kb HindIII-BamHI fragment containing the ubiquitin promoter (Toki, et al. Plant Physiol. 100: 1503) was cloned into the HindIII - BamHI sites of this clone and the nos terminator was ligated into the NotI-SacI sites. This generated plasmid pCIB7712. This placed the maize PR1 leader-lactoferricin B-vacuole target sequence under control of the ubiquitin promoter and the nos terminator (ubi-PR1-lactoB-vac-nos). A DNA fragment comprised of ubi-SynPAT-nos was ligated into the HindIII site of this plasmid, resulting in plasmid pCIB7713.

pCIB7713 is cleaved with KpnI and SacI to release the insert containing the ubi-SynPAT-nos -- ubi-PR1-lacto-vac-nos gene fusions. This fragment is cloned into KpnI, SacI digested pCIB6848 to form pCIB7714. This plasmid contains the NPTII (kanamycin resistance) gene for selection in bacteria and the SynPAT gene for selection in plants, and is useful for transformation of plants with the lactoferricin B gene under control of the maize ubiquitin promoter.

The KpnI-SacI fragment from pCIB7712, containing the ubi-lacto-nos cassette is cloned into plasmid pCIB6848, to generate pCIB771015. This places the ubi-lacto-nos gene construction in a kanamycin resistance gene containing plasmid.

Example 6: Construction of a gene for plastid expression of lactoferricin B in plants

For plastid expression of lactoferricin B, the chloroplast target sequence of the ribulose-1,5-biphosphate carboxylase small subunit (ssu) gene is cloned and placed 5' to the lactoferricin B gene sequence. This transit peptide sequence directs the small subunit protein to the chloroplast, where it is cleaved off, to release the mature protein. The designed transit peptide-lactoferricin B gene constructs contain (5' to 3') a BamHI restriction site, a Kozak consensus sequence, including the methionine start codon, a chloroplast target sequence fused to the lactoferricin B coding sequence, a stop codon and a NotI

restriction site. Lactoferricin B gene constructs are made with the chloroplast target sequence of the ssu gene from Arabidopsis (ats1A; Wong et al. Plant Mol. Biol. 20, 81-93, 1992) and from maize (Matsuoka et al., J. Biochem. 102, 673-676, 1987). The coding sequence of the transit peptide of these ssu genes (Wong et al., Plant Mol. Biol., 20, 81-93, 1992) is cloned by PCR amplification from genomic DNA, cDNA or a cDNA library. A series of three transit peptide sequences (TP1, TP2, and TP3) are constructed from both the Arabidopsis and maize ssu genes, following the design of Wong et al. (Plant Mol. Biol. 20, 81-93, 1992). Thus, TP1 contains the coding sequence of the transit peptide only (up to the cleavage site), whereas TP 2 and 3 have the entire coding sequence for the transit peptide and part of the coding sequence of the mature ssu protein, with a duplicated cleavage site. TP2 has an additional 2 amino acid codons past the duplicated cleavage site. TP3 ends exactly at the duplicated cleavage site.

a. Arabidopsis chloroplast target sequence-lactoferricin B gene constructs

The first transit peptide sequence was cloned by using oligonucleotides ATP-5 'and ATP1-3' for the amplification. The sequences for these oligonucleotides were:

ATP-5' (SEQ ID NO:16):
5'-AGTAGGATCCACCATGGCTTCCTCTATGCTC-3'

ATP1-3' (SEQ ID NO:17):
5'-GCAGTTAACTCTTCCGCCGTT-3'

The ATP-5' oligonucleotide contained the BamHI restriction site to facilitate cloning, a Kozak consensus sequence 5' to the initiation codon and the first 18 nucleotides of the coding sequence of the ats1A gene. Genomic DNA was used as template. The amplified product was cloned into pCR-Script(SK+) and clones were sequenced to verify that the insert contained 165 bp of the ats1A transit peptide coding sequence, extending from +1 to +165 bp. A clone with the correct sequence was called pATP1.

Fusion of the transit peptide sequence to lactoferricin B was achieved by PCR using 4 primers:

ATP-LF 5' (SEQ ID NO:18): 5'-GTAGGATCCACCATGGCT-3', from the 5' end of the ATP1 amplification product;

ATP-LF 3': identical to oligo 2-1 described in Example 3 above;

and 2 bridge primers spanning the ats1A/lactoferricin B fusion and consisting of the 18 nucleotides at the 3' end of ATP1 followed by the 18 nucleotides at the 5' end of the lactoferricin B coding sequence. The sequence of 5' bridge primer was:

ATP1-LFB 5' (SEQ ID NO:19): 5'-GGCGGAAGAGTTAACTGCTTCAAGTGCCGCCGCTGG-3'
The sequence of 3' bridge primer (the complement of the 5' bridge primer) was:

ATP1-LFB 3' (SEQ ID NO:20): 5'-CCAGCGGCGGCACTTGAAGCAGTTAACTCTTCCGCC-3'

For each transit fusion construct, two rounds of amplification were performed. The first round involved two sets of reactions. Reaction 1 generated specific ATP1 PCR fragments with a short lactoferricin B sequence extension. This reaction included primers ATP-LF 5' and ATP1-LFB 3' and plasmid pATP1 DNA. Reaction 2 generated lactoferricin B PCR fragments with a short 5' ATP1 sequence extension. This reaction included primers ATP-LF 3' and either ATP1-LFB 5' using cloned lactoferricin B as template. The second round of amplification was performed by mixing the products of reactions 1 and 2 in equal volumes, denaturing for 1 min at 94°C, then extending the annealed products for 5 min. at 72°C in the presence of Taq DNA polymerase and nucleotides to form "full-length" template. This was followed by addition of primers ATP-LF 5' and ATP-LF 3' and PCR amplification.
This generated the ATP1-lactoferricin B gene fusion. The PCR products were cloned into the PCRII vector.
Clones are isolated and sequenced. Plasmids with the correct sequence are digested with BamHI and Not I and the insert cloned into BamHI, NotI predigested Bluescript. A 2 kb HindIII-BamHI fragment containing the ubiquitin promoter (Toki, et al. Plant Physiol. 100: 1503) is cloned into the HindIII - BamHI sites of this clone and the nos terminator is ligated into the NotI-SacI sites. The resulting plasmid contains the ATP1 chloroplast target sequences upstream of the lactoferricin B coding sequence. This gene construct is positioned between the ubiquitin promoter and the nos terminator.
A DNA fragment containing the ubi-SynPAT-nos gene construct is cloned into the HindIII site of these plasmids, to generate pCIB7721. The resulting plasmids are cleaved with KpnI

and SacI to release the insert containing the ubi-SynPAT-nos -- ubi-ATP1/2/3-lacto-nos gene fusions. These fragments are cloned into KpnI, SacI digested pCIB6848 to form pCIB7722. This plasmid contains the NPTII (kanamycin resistance) gene for selection in bacteria and the SynPAT gene for selection in plants, and is useful for transformation of plants with the lactoferricin B gene under control of the maize ubiquitin promoter.
The KpnI-SacI fragment from pCIB7720, containing ubi-ATP1-lacto-nos gene fusions are cloned into plasmid pCIB6848, to generate pCIB7723. This places ubi-ATP1-lacto-nos gene fusions in a kanamycin resistance gene containing plasmid.

The second transit peptide coding sequence, ATP2, is cloned in a similar manner as ATP1, using oligonucleotides ATP-5' and ATP2-3', using genomic DNA or oligo dT-primed, reverse transcribed RNA as template.

Oligo ATP2-3' sequence (SEQ ID NO:21):

5'-CTGCATGCAGTTGACGCGACCACCGGAATCGGTAAGGTCAGG-3'

The PCR products are cloned and clones with insert are sequenced to verify that it contains 261 bp of the ats1A transit peptide coding sequence, extending from +1 to +261. A clone with the correct sequence is called plasmid pATP2.
The third transit peptide coding sequence, ATP3, is cloned in a similar manner as ATP2, using oligonucleotides ATP-5' and ATP3-3'.

Oligo ATP3-3' sequence (SEQ ID NO:22):

5'-GCAGTTGACGCGACCACCGGAATCGGTAAGGTCAGG-3'.

The PCR products are cloned and a clone with the correct sequence is called pATP3, containing 255 bp of the ats1A transit peptide coding sequence, extending from +1 to +255.

These transit peptide sequences are fused to the lactoferricin B gene sequences as described above for ATP1.
The sequences of the set of 5' bridge primers are:

ATP2-LFB 5' (SEQ ID NO:23):

5'-CGCGTCAACTGCATGCAGTTCAAGTGCCGCCGCTGG-3'

ATP3-LFB 5' (SEQ ID NO:24):

5'-GGTGGTCGCGTCAACTGCTTCAAGTGCCGCCGCTGG-3'.

The sequences of the set of 3' bridge primers (the complement of the 5' bridge primers) are:

ATP2-LFB 3' (SEQ ID NO:25):

5'-CCAGCGGCGGCACTTGAACTGCATGCAGTTGACGCG-3'

ATP3-LFB 3' (SEQ ID NO:26):

5'-CCAGCGGCGGCACTTGAAGCAGTTGACGCGACCACC-3'.

The resulting PCR fusion products are cloned. Clones with the correct sequence have ATP2-lactoferricin B and ATP3-lactoferricin B gene fusions. These fragments are cloned into Bluescript and the ubiquitin promoter and nos terminator are added as described above to generate pCIB7730 and pCIB7740. The ATP1/2/3-lactoferricin B gene constructs are positioned between the ubiquitin promoter and the nos terminator. New constructs with the ubiquitin-SynPat-nos fragment are generated as described above for pCIB7720, resulting in plasmids pCIB7731 and pCIB7741.

b. Maize chloroplast target sequence-lactoferricin B gene constructs

Additional transit peptide-lactoferricin B fusion constructs are synthesized using transit peptide sequences of a maize ssu gene (Matsuoka et al., J. Biochem. 102, 673-676, 1987) in the same manner as described for the Arabidopsis ssu transit peptide-lactoferricin B gene constructs. The only differences are the source of template DNA for the generation of the transit peptides and the oligonucleotide sequences.
The template DNA for the PCR amplification of the maize ssu transit peptide sequence is maize genomic DNA, cDNA or a cDNA library. The oligonucleotide sequences (5' to 3') are as follows:

MTP-5' (SEQ ID NO:27):

5'-AGTAGGATCCACCATGGCGCCCACCGTGATG-3'

MTP1-3' (SEQ ID NO:28):

5'-GCACCGGATTCTTCCGCCGTT-3'

MTP2-3' (SEQ ID NO:29):

5'-CTGCATGCACCGTATGCGACCACCGTCCGTCGACAGCGGCGG-3'

MTP3-3' (SEQ ID NO:30):

5'-GCACCGTATGCGACCACCGTCCGTCGACAGCGGCGG-3'

MTP-LF 5' (SEQ ID NO:31):
5'-GTAGGATCCACCATGGCG-3'

MTP-LF 3' (SEQ ID NO:32):
5'-GCGGCCGCTTAGAAGGC-3'

MTP1-LFB 5' (SEQ ID NO:33):
5'-GGCGGAAGAATCCGGTGCTTCAAGTGCCGCCGCTGG-3'

MTP2-LFB 5' (SEQ ID NO:34):
5'-CGCATACGGTGCATGCAGTTCAAGTGCCGCCGCTGG-3'

MTP3-LFB 5' (SEQ ID NO:35):
5'-GGTGGTCGCATACGGTGCTTCAAGTGCCGCCGCTGG-3'

MTF1-LFB 3' (SEQ ID NO:36):
5'-CCAGCGGCGGCACTTGAAGCACCGGATTCTTCCGCC-3'

MTF2-LFB 3' (SEQ ID NO:37):
5'-CCAGCGGCGGCACTTGAACTGCATGCACCGTATGCG-3'

MTF3-LFB 3' (SEQ ID NO:38):
5'-CCAGCGGCGGCACTTGAAGCACCGTATGCGACCACC-3'

The plasmids that are generated contain the maize transit peptide sequences (MTP1, 2 and 3) fused to the lactoferricin B coding sequence.

Example 7: Lactoferricin B gene expression in maize protoplasts

Protoplasts which were known to allow transient expression were used for transient expression of lactoferricin B containing plasmids. Protoplasts were isolated from suspension cultured cells as described in Shillitto et al. (US patent 5,350,689: Zea mays plants and transgenic Zea mays plants regenerated from protoplasts or protoplast derived cells. 1989) and resuspended in 0.5M mannitol/15mM $MgCl_2$ at a concentration of $20x10^6$ per ml. Plasmid DNA used for transformations were: pUbi-bar, containing the Ubiquitin promoter plus first exon and intron, the bar gene and the nos terminator; pUbi-GUS, containing the

Ubiquitin promoter plus first exon and intron, the GUS gene and the nos terminator; and pCIB7703.

Plasmid DNAs were introduced into the protoplasts by PEG precipitation (Kramer et al., Planta 190, 454-458, 1993) as follows: protoplasts were heat-shocked by gently agitating the tube in a 45°C water bath for 4 min. Aliquots of 500 microliter, containing 10 million protoplasts were transferred to sterile tubes and the following additions made to each tube: 25-50 µg of each plasmid and 450 microliter 40% PEG. The PEG was prepared from PEG 8000 (Sigma Chemical Co., St. Louis, MO) dissolved in a solution of 0.4M mannitol and 0.1M $Ca(NO_3)2 \bullet 4H_2O$, with the pH adjusted to 8.0. Tubes were allowed to stand for 20 min. with occasional gentle swirling, then diluted at 5 min. intervals with 1 ml, 2 ml and 5 ml volumes of W6 solution (5mM KCl, 61mM $CaCl_2 \bullet H_2O$, 154mM NaCl, 51mM glucose, 0.1% 2-(N-morpholino) ethanesulfonic acid, pH 6.0). After the final dilution, protoplasts were centrifuged for 10 min at 60g-100g and most of the supernatant removed, leaving about 0.5 ml of liquid over the protoplasts. Protoplasts were resuspended in the remaining liquid by gentle shaking. Protoplasts were resuspended at a density of two million/ml in FW medium and cultured at room temperature in the dark for 18 h after DNA introduction for analysis of RNA and protein expression.

Protein extracts were prepared from aliqouts of all transformations for subsequent GUS activity determinations, according to the manufacturer's specifications (Tropix). The results showed that cells transformed with a combination of pCIB7703 and ubi-GUS had abundant GUS activity ($10x10^6$ counts/min/$1x10^6$ protoplasts), comparable to the GUS activity in cells transformed with ubi-bar and ubi-GUS ($10x10^6$ counts/min/$1x10^6$ protoplasts). This indicated that expression of the lactoferricin B gene construct had no effect on GUS gene expression in the transiently expressing cells, i.e. no cell toxicity was evident. Cells transformed with ubi-bar and pCIB7703 showed only background levels of GUS activity (5000 counts/min/$1x10^6$ protoplasts).

RNA samples were prepared for reverse transcription-PCR (RT-PCR) analysis to evaluate the presence of lactoferricin B mRNA. For this purpose, total and poly(A) RNA was isolated from transfected protoplasts. RT-PCR was performed using a commercial kit (Stratagene), per manufacturer's instructions. For detection of ubi-lactoferricin B-nos cDNA, PCR primers were used that were complementary to the sequence just 5' to the polyadenylation signal of the nos terminator and to the first exon in the ubiquitin promoter. Since there was an intron in the pCIB7703 construct between this exon and the lactoferricin

B coding sequence/nos terminator, the resulting PCR band could be verified as being derived from RNA, based on its size.

For the RT-PCR reaction, 1 µl of total RNA was reverse transcribed according to the manufacturer's instruction, in a 50 µl reaction volume. Of this, 1 µl was used for PCR amplification using primers 176 (SEQ ID NO:39: 5'-TTCCCCAACCTCGTGTTGTTC-3') and 179 (SEQ ID NO:40: 5'-CCAAATGTTTGAACGATCGCG-3'), or primers 177 (SEQ ID NO:41: 5'-CACAACCAGATCTCCCCCAAA-3') and 180 (SEQ ID NO:42: 5'-AAATTCGCGGCCGCTTAGAAG-3'). Reaction conditions were: 45 sec. at 94°C, 45 sec. at 60°C and 2 min. at 72°C, for 43 cycles. The samples were size separated on agarose gel. The primers 176 and 179 yielded a PCR fragment of approximately 220 bp, as expected if template DNA was cDNA derived from cells that were transformed with the pCIB7703 plasmid. A PCR band derived from plasmid was expected to be 1.3 kb in size.

For northern analysis, RNA samples are size separated on an agarose gel and blotted onto a nylon membrane. This blot is subsequently hybridized to a radioactive probe containing lactoferricin B gene construct sequences.

Protein extracts for immunoblotting are prepared by making a protoplast suspension in an appropriate buffer. The suspension is microcentrifuged for five minutes and the supernatant and pellets are suspended in Laemmli sample buffer and subjected to Laemmli SDS gel electrophoresis. Since disulfide linkage of lactoferricin B peptide to proteins may be a problem, reduction with DTT or 2-ME, followed by iodoacetamide blockage of protein -SH groups in extracts may be required before electrophoresis.

Electrophoresed gels are electroblotted to nitrocellulose for Western analysis, using an anti-lactoferricin B antiserum.

Protein extracts for mass spectroscopy are prepared by vortexing the protoplast suspension vigorously in a buffer, microcentrifuging twice for 10 minutes after which the supernatant is collected each time. An appropriate dilution is analyzed by MALDI-MS (Voyager, Perceptive Biosystems), using standard protocols for peptide analysis. If the samples need to be further purified, extracts can be fractionated on a C18 column. Fractions are then analyzed for the presence of the lactoferricin B peptide by MALDI-MS.

Protein extracts are also prepared for evaluation of antifungal activity. In this case, the protoplasts are homogenized in 20mM Tris buffer, pH 7.5, containing 1.5% PVPP, 5mM DTT, and 10 µM AEBSF (4 - (2-Aminoethyl) - benzenesulfonyl fluoride, hydrochloride). The

extracts are evaluated for antifungal activity based on in vitro fungal inhibition assays as described in Example 1 above.

Transient expression analysis of other lactoferricin B gene containing constructs is performed using methods described in this Example.

Example 8: Plant transformation, selection and regeneration

a. Description of plasmids and selectable markers

The lactoferricin B gene constructs are under control of the ubiquitin promoter and the nos terminator. For corn transformation, a selectable marker is cloned into these plasmids, consisting of the synthetic phosphinothricin acetyltransferase gene (designated SynPAT), under control of the maize ubiquitin promoter and the nos terminator (ubi-SynPAT-nos). SynPAT is a synthetic version of the bar gene from a Streptomyces strain (Thompson et al., EMBO 6:2519-2523, 1987), that was optimized for expression in plants (US patent 5,276,268: Phosphinotricin-resistance gene and its use). The plasmid containing the ubi-SynPAT-nos cassette (pUBIAc) was obtained from Hoechst Aktiengesellschaft, Frankfurt, Germany. The plasmids containing ubi-lactoferricin B-nos and ubi-SynPAT-nos cassettes are used to transform corn. The presence of the SynPAT gene cassette allows selection of transformed plants using the herbicide BASTA.

For wheat transformation, plasmids containing ubi-lacto-nos gene cassettes are co-bombarded with a plasmid (pUBA or pUBA/kan) containing the bar selectable marker gene under control of the ubiquitin promoter and nos terminator (Toki et al, 1992 Plant Physiol. 100:1503-1506). The presence of the bar gene cassette allows selection of transformed plants with the herbicide BASTA. The pUBA plasmid contains ubi-bar-nos and the ampicillin resistance gene for selectioin in E. coli. Plasmid pUBA/kan contains a kanamycin resistance gene instead of the ampicillin resistance gene for selection in E. coli.

A hygromycin gene from E. coli (Gritz and Davies, Gene 25, 179-188, 1983) was isolated, Bgl II linkers were ligated onto the ends and this fragment was next cloned into the BamHI site of vector pCIB710, to generate pCIB712. Two oligonucleotides (5'-AGTAGGATCCATGAAAAAGCCTGAACTC-3' (SEQ ID NO:43) and 5'-TACTGGATCCCTATTCCTTTGCCCTC-3' (SEQ ID NO:44)) were used to PCR amplify the gene from pCIB712. The PCR fragment was digested with BamHI and cloned into the BamHI site of pPEH3. Clones with insert were sequenced and one with the correct sequence and the correct orientation of the gene was named pCIB7613. This plasmid

contained the hygromycin resistance gene under control of the ubiquitin promoter and the nos terminator. Following bombardment of plants with this plasmid, transformed plants can be selected using hygromycin as the selection agent.

The acetohydroxyacid synthase (AHAS) gene was cloned from a Lambda-Zap genomic library derived from sulfonylurea (Beacon) resistant maize tissue, using as a probe the Arabidopsis gene (K. Sathasivan et al., Nucl. Acid Res. 18, 2188). A G to A transition was introduced at position 1862 in this gene, by PCR amplification, using one oligonucleotide with the appropriate single base change. This nucleotide change resulted in a Ser to Asn change. This gene was cloned into a vector to generate plasmid pCIB4247. PCR amplification using 2 oligonucleotides specific for the start and end of the maize AHAS gene in pCIB4247 (5'-TATCTCTCTCTATAAGGATCCATGGTCACC-3' (SEQ ID NO:45) and 5'-TACTGGATCCTCAGTACACAGTCCTGCC-3' (SEQ ID NO:46)) generated fragments that were cloned into pCRII. Inserts were sequenced to verify the absence of mutations and a plasmid with a correct insert was used to isolate the BamHI fragment with the AHAS sequence. This fragment was cloned into the BamHI site of pPEH3, to generate pCIB7612. This plasmid contains a mutated maize AHAS gene under control of the maize ubiquitin promoter and the nos terminator. This gene confers resistance to imidazolinone (imazaquin/Scepter) when transformed into plants.

The protoporphyrinogen oxidase (protox) cDNAs from Arabidopsis and maize and the protox gene promoter from Arabidopsis were isolated. The protox cDNAs from Arabidopsis and maize were cloned by complementation of a protox deficient E. coli strain. Mutant Arabidopsis and maize protox genes were selected that resulted in increased resistance to the protox inhibiting herbicide. The herbicide resistant Arabidopsis protox cDNA was cloned into a plasmid between the double 35S promoter and the tml terminator.

The Arabidopsis protox promoter was isolated from a genomic library, using a fragment from the 5' end of the Arabidopsis protox cDNA as a probe. The plasmid with the promoter was digested with NcoI and BamHI, leaving the promoter attached to the vector, but removing the downstream sequences. The NcoI-BamHI fragment containing the herbicide resistant protox cDNA and tml terminator was cloned into it. This created a plasmid with approximately 600 nucleotides of the Arabidopsis protox promoter, the coding sequence of the herbicide resistant Arab protox gene and the tml terminator. Plants transformed with this plasmid can be selcted on medium containing the herbicide.

b. Transformation of lactoferricin B gene constructs into maize

The method used for maize transformation has been described by Koziel et al. (Biotechnology 11, 194-200, 1993) using particle bombardment into cells of immature embryos. Immature embryos of maize inbred CGA00526 were isolated aseptically approximately 10 days after pollination, and plated scutellum side up on modified Duncan's "D" medium (Duncan et al., Planta 165, 322-332, 1985). The medium was supplemented with Chloramben (5mg/L) instead of dicamba. Approximately 2 weeks later, callus was isolated from the embryos and plated on media supplemented with 2,4-dichlorophenoxy acetic acid (5mg/L) instead of Chloramben and subsequently subcultured at 10 - 14 day intervals. Four to six hours prior to transformation, 1 - 4 month old callus was transferred to fresh medium containing 12% sucrose as an osmoticum.

Plasmid DNA, or isolated fragment DNA was precipitated onto gold particles as described in the DuPont Biolistic manual. Each plate of tissue was shot twice with the DuPont Biolistics helium device. A rupture pressure of 600 - 1100psi and a standard 80 - 100 µm mesh screen were used. After bombardment, the tissue was placed in the dark for 12 - 24 hours. Following this time period, the tissue was transferred back to modified Duncan's "D" medium with the addition of the selection agent BASTA at a minimum of 20 mg/L and grown in the dark. The modified Duncan's "D" medium has the amino acid supplement replaced with modified Koa's amino acid supplement (K. W. Kao and M. R. Michayluk, Planta 126, 105-110, 1975), except that glutamine and asparagine are omitted completely. The tissue was transferred to fresh media containing BASTA at a minimum of 20 mg/L, at no less than 14 day intervals for 60 - 80 days. During this subculture period nonembryogenic callus was removed.

After this period of selection in the dark, the tissue was transferred to an MS based medium (Murashige and Skoog, Physiol. Plant 15, 473-439, 1962) containing the following hormones to induce germination and development of somatic embryos: ancimidol (0.25mg/L), kinetin (0.5mg/L), napthaline acetic acid (1mg/L), and BASTA at a minimum of 5 mg/L. The tissue was cultured on this medium in a 16 hour light regime for 10 - 14 days, then subcultured to MS based media with BASTA (3 mg/L) minus the plant hormones to allow plantlet development. As plantlets developed, they were transferred to a 3/4 strength MS medium and allowed to develop roots. Rooted plants were transferred to soil and grown in the greenhouse.

c. Transformation of lactoferricin B gene constructs into wheat

Transformation of immature embryos and immature embryo-derived callus using particle bombardment has been described by Vasil et. al. (Biotechnology 11: 1553-1558, 1993) and Weeks et. al. (Plant Physiology 102: 1077-1084, 1993).
Immature embryos of spring or winter wheat are isolated aseptically approximately 2 weeks after pollination, and cultured scutellum side uppermost on MS based medium (Murashige and Skoog, 1962 Physiol. Plant 15:473-439) supplemented with 2,4-D (dichloropheoxyacetic acid), glutamine and asparagine for callus induction for 6-10 days. Four to six hours prior to transformation embryos are selected for an embryogenic response and transferred to fresh medium also containing 15% maltose as an osmoticum. Constructs used for transformation include a plasmid containing the ubi-bar-nos cassettte (pUBA or pUBA/kan) allowing selection of transformed tissues with BASTA. Alternatively, constructs containing genes conferring resistance to other herbicides (e.g., sulfonylureas, imidazolinones) or antibiotics (e.g., hygromycin), can be used. This construct is co-transformed with one of the lactoferricin B containing gene constructs described in Examples 2-6 above, including pCIB7706, pCIB7710, pCIB7715, pCIB7723, pCIB7733 and pCIB7743. For this, plasmid DNA is precipitated onto gold particles as described in the Dupont Biolistic manual. Each plate of embryos is shot with the DuPont PDS1000 helium device using a burst pressure of 1100psi and a standard 80 mesh screen. After 24 hours, the embryos are removed from the osmoticum and placed back onto induction media. Approximately one month later, the embryo explants with developing embryogenic callus are transferred to regeneration medium (MS + 1mg/L napthaline acetic acid and 5mg/L gibberellic acid), containing 1 mg/L BASTA for two weeks, then transferred to hormone free regeneration media containing 3 mg/L BASTA for 2 to 6 weeks, until shoots develop. The embryos with shoots are transferred to half strength MS with 0.5mg/L NAA and 3 mg/L BASTA until roots develop, at which time the plantlets are transferred to soil and grown to maturity. Plant tissue is harvested for analysis (described in Example 10) and plants are selfed for production of seed.

d. Transformation of lactoferricin B gene constructs into sugar beet

Guard cells are isolated from sugarbeet leaf sections without midribs, as described in Hall et al, Nature Biotechnology, vol. 14, pages 1133-1138, 1996. The leaf sections are homogenized with a blender (23,000 rpm for 60 seconds) in 50 ml Ficoll medium (100g/l Ficoll, 735 mg/l calcium chloride · 2 H_2O and 1 g/l PVP40) while kept on ice. Following

filtration through a nylon mesh (300 micrometer), the homogenate is washed with 500 ml of cold water and transferred to 10 ml CPW9M containing 3.8% (w/v) calciumchloride · 2 H_2O. The epidermis is then recovered by centrifugation and digested with cell wall degrading enzymes (0.5% cellulase and 3% macerozyme, Yakult Honsha, Japan) for 16 hours to release individual cells. Following filtration through 55 micrometer filters, the suspension is mixed with an equal volume of Percoll containing 15% sucrose. In tubes, 1 ml CPW15S is layered on top of the protoplast suspension, followed by 0.5 ml 9% mannitol/1mM calciumchloride. The guard cells are recovered from the top layer after a 10 minute spin at 55g and reisolated once again in the same way.
DNA (50 microgram per one million cells) is added to guard cells resuspended in 0.75 ml 9% mannitol/1 mM calciumchloride (mannitol solution), and this is followed by the addition of 0.75 ml 40% PEG 6000. After 30 minutes at room temperature, 2 ml aliquots of F medium is added every 5 minutes, for a total of 4 aliquots. Protoplasts are washed with the mannitol solution and then embedded in Ca alginate. They are cultured in modified K8P medium and selection with bialaphos is started one week later at 200 microgram/liter. Eleven days later, small alginate sections are embedded in agarose, and cultured in PG1B medium with 250 microgram bialaphos/liter. Calli that develop are transferred to fresh plates with bialophos and cultured for 2 weeks. After this they are cultured without selection, at 25°C, with a light/dark regimen (16h/8h). Subculturing is performed every 2 weeks and plantlets are regenerated in 4 weeks. They are rooted and planted in soil.

Example 9: Analyses of transgenic plants expressing lactoferricin B

Tissue from transformed plants that survive the selection process (Example 8) are analyzed for the presence of the lactoferricin B gene construct (PCR), RNA derived from this transgene (northern blot hybridization, RT-PCR) and protein derived from this transgene (Western).

a. <u>PCR analysis</u>

DNA is extracted from transformed plant tissue using the IsoQuick nucleic acid extraction kit (ORCA Research Inc.) according to instructions provided with the kit. PCR analyses are performed according to standard protocols. The primers used for amplification of the lactoferricin B gene constructs are designed from the known sequences of the transgene constructs.

b. Northern analysis

Transformed plants were analyzed for the presence of RNA by northern blot hybridization. For northern blot analysis, RNA was extracted from leaf tissue. Following grinding of the frozen tissue, extraction buffer was added (0.1 M LiCl, 100 mM Tris pH 8, 10 mM EDTA, 1% SDS), followed by equal volumes of water-saturated phenol and chloroform. The RNA was precipitated from the aqueous phase with sodium acetate/ethanol and size separted on a formaldehyge containing agarose gel. The gel was blotted to GeneScreen Plus nylon, which was hybridized to a probe derived from the lactoferricin B gene. Following overnight hybridization, the blot was washed at high stringency (65°C) and exposed to X-ray film.

c. RT-PCR analysis

RNA samples are also analyzed by RT-PCR for the presence of lactoferricin B RNA, as described in example 7 above.

d. Western analysis

Transformed plants were tested for the presence of lactoferricin B or lactoferricin B-derivative proteins encoded by the transgene. For western analysis, fresh leaves were snap frozen in liquid nitrogen and ground to a fine powder. The samples were then treated with cold 10% trichloroacetic acid in acetone at -20°C for 1 hr, spun down, washed with cold acetone and air-dried. The dried samples were then incubated with 0.05 M sulfuric acid (3 ml/g fresh weight) for 1 hr on ice. The extracts were neutralized with 0.01 N sodium hydroxide, and dried. Samples were run on 10%-20% Tricine SDS gels (Novex) according to manufacturer's instructions. Proteins were transferred to nitrocellulose at a constant current of 200 mA for 3 hr in a wet cell, then stained with Ponceau Red to visualize membrane-bound protein.

Proteins transferred to nitrocellulose were probed with immuno-affinity purified goat anti-lactoferricin-B antibodies, followed by secondary and alkaline phosphatase-conjugated tertiary antibodies. Anti-lactoferricin B reactive bands were detected after addition of a nitro blue tetrazolium / bromo-chloro-indolyl phosphate substrate solution.

e. Mass spectroscopy

Protein extracts prepared from transformed plants are also analyzed for the presence of the lactoferricin B peptide, using a MALDI-MS, as described in Example 7 above.

f. Evaluation of antimicrobial activity in lactoferricin B transgenic plant extracts

Protein extracts are prepared from lactoferricin B transgenic plants by homogenizing tissue in 20mM Tris buffer, pH 7.5, containing 1.5% PVPP, 5mM DTT, and 10 µM AEBSF (4 - (2-aminoethyl) - benzenesulfonyl fluoride, hydrochloride). Protein extracts are further purified by established protocols such as ammonium sulfate precipitation, HPLC chromatography and immuno purification using an anti-lactoferricin B antiserum. The protein concentration is determined in each extract and aliquots are used in antifungal assays.

The three assays described in Example 1 above are used to evaluate the presence of antimicrobial activity in the crude and partially purified protein extracts. The organisms used to test for the presence of antimicrobial activity include those listed in Example 1, as well as other important fungal pathogens of corn and wheat and lactoferricin B sensitive bacterial lab strains, such as E. coli and Staphylococcus aureus (W. Bellamy et al., J. Appl. Bacteriol. 73, 472-479, 1992).

Example 10: Testing Lactoferricin B transgenic plants for increased disease resistance

a. Testing of transgenic maize plants

1. Southern corn leaf blight (SCLB) assay

SCLB is caused by Bipolaris maydis, formerly named Cochliobolus heterostrophus and Helminthosporium maydis. B. maydis cultures are grown on PDA (potato dextrose agar) under continuous near-UV light to induce sporulation. Spores are harvested by gently scraping the plate using a glass rod in 1% Tween-20. The solution is collected and the spore concentration adjusted to 500-1000 spores/ml with double distilled, sterile water. Transgenic plants and wild type control plants are grown in a greenhouse to 3-5 weeks of age. The 3 upper fully expanded leaves are sprayed with the spore suspension, by applying
a fine mist of the spore suspension to each leaf, using an aerosol sprayer. Next, the plants

are placed in a closed chamber and incubated overnight (16-20 hours) in a mist chamber, under high humidity conditions. The plants are moved from the mist chamber into a growth chamber. Approximately one week after inoculation, lesions that develop are counted and the size of 10-15 representative lesions are measured and compared between the control and transgenic plants. Plants are also visually rated for disease progression, at 3-4 day intervals over a 14 day period following inoculation.

Plants from a T1 line, B10b, that expressed Met-Lactoferricin B mRNA showed increased disease resistance, as disease symptoms that were less than those of the transgenic controls and wild type controls.

2. Anthracnose leaf blight

Colletotrichum graminicola is the causal agent of anthracnose leaf blight. It causes necrosis of infected leaves. For the disease assays, spores were collected from sporulating agar plates as described for B. maydis. Leaves of 3 to 5 week old plants were sprayed with a spore suspension at approximately 10E6 spores/ml concentration. The inoculated plants were incubated overnight in the mist chamber, after which they were moved to a growth chamber, as described for B. maydis infected plants. Over a 2 week period, at 3-4 day intervals, the percentage of leaf area that was necrotic was determined and compared between transgenic and non transgenic controls.

3. Carbonum leaf spot assay

Helminthosporium carbonum is the causal agent of maize carbonum leaf spot. For the disease assay, spores were harvested from sporulating cultures, as described for B. maydis. Leaves of 3-5 week old corn plants were sprayed with the spore suspension at an concentration of 2.5 to 10 x 10E4/ml, and the plants were then incubated in the mist chamber overnight. Next, the plants were grown for approximately 14 days in the growth chamber. Disease progression was followed, as described for anthracnose leaf blight.

Plants from T1 line B10b that expressed RNA for Met-lactoferricin B showed increased disease resistance. In the expressing plants, decreased disease symptoms were observed following carbonum leaf spot inoculation, compared to transgenic controls and wild type controls.

4. Fusarium root assay

Fusarium moniliforme causes Fusarium ear mold on corn ears. F. moniloforme is grown on PDA plates and spores harvested from the plates as described for B. maydis. Perforated filter paper is folded, placed in a pouch, and surface sterilized corn seeds placed in the fold, such that the emerging roots grow through the perforations to the bottom of the pouch. A 12 ml spore suspension of F. moniliforme is placed in the bottom of the pouch and the pouch incubated in a growth chamber at 25-30°C. Inhibition of root growth and root necrosis due to fungal infection is assayed 7-14 days later and compared between transgenic and non transgenic controls.

5. Pythium root assay

Pythium aphinadermatum is one of the causal organisms involved in maize stalk rot and root rot. For disease assays, a pouch format is used, as described for F. Inoculum is prepared from zoospores or mycelium. For zoospore formation, mycelia are placed in contact with leaf pieces of tall fescue grass, incubated under continuous fluorescent light and zoospores that develop are harvested. Alternatively, potato dextrose medium is inoculated with a mycelial plug from a Pythium PDA plate and allowed to grow for 2-3 weeks. The mycelia are removed from the culture and dispersed using a blender, in sterile double distilled water.

Corn seeds are placed in the filter paper fold, as described and inoculum (zoospores or dispersed mycelium, at an appropriate concentration) added to the bottom of the pouch. As described for F. moniliforme, after incubation for 1-2 weeks, root length and necrosis is assayed and compared between transgenic and non transgenic controls.

6. Stewart's bacterial wilt

Erwinia stewartii causes Stewart's wilt on corn plants. For disease assays, bacteria are grown to high density in Nutrient medium and diluted to approximately 10^7 bacteria/ml. Leaves of corn plants are infected with the bacteria by pricking the leaves with needles that are dipped in the bacterial suspension. Lesions are measured 1, 2 and 3 weeks after inoculation.

7. Leaf disc assays

The whole-plant disease assays described above can also be performed on leaf discs that are placed in moist petridishes.

b. Testing of transgenic wheat plants

1. Pseudomonas disease assay

Pseudomonas syringae pv. syringae Van Hall causes bacterial leaf blight of wheat. For the disease assay in which visual disease symptoms are scored, the bacteria (strain BL882) are grown overnight on L-broth agar plates. A small swath of cells is scraped from the plate with a toothpick and resuspended into a solution of 10 mM $MgCl_2$. The absorbance of the suspension at 600 nm is taken in order to determine the concentration of bacteria in the suspension, which is calculated as 1 x 10^8 cfu/ml per OD600 of 0.1. The culture is diluted to 1x10^7 bacteria/ml and small amounts are drawn by suction into a 1 ml plastic pasteur pipet. Approximately 50 ul of bacteria are injected into the leaves of 3 week old wheat plants. The extent of the inoculated area is lightly marked using a black felt-tip pen. The plants are placed in a plastic box with the internal environment humidified by heavy application of water to the plants using a common garden sprayer. The box is closed tightly and placed in a Percival chamber at 18-20°C and given 16 hours of light per day. After five days, the severity of disease is scored by calculating the percentage of lesioned area, as evidenced by dark necrotic patches surrounded by a thin layer of chlorosis, occurring within the inoculated zone.

For more precise quantification of the degree of disease development, leaves are evenly inoculated with BL882 containing a plasmid with the kanamycin resistance gene, by infiltration of bacteria at a concentration of 1x10^5 cfu/ml. On the day of infiltration and on consecutive days, the bacterial titer in the injected area is determined. This is accomplished by combining leaf punches from infiltrated areas that collectively cover a 1 cm space, grinding them in 10 mM $MgCl_2$, and spreading dilutions of the extract on LB plates supplemented with 50 microgram/ml kanamycin (this BL882 isolate has been transformed with a kanamycin resistance gene). After three days of growth, single bacteria from the leaf grow into colonies which are easily counted. In this manner, the growth rate of the bacteria in transformed plants can be quantified and compared to that of control, untransformed plants.

2. Septoria nodorum assay

S. nodorum causes causes leaf blotch on wheat leaves and glume blotch on wheat ears. For the disease assay, spore are collected from sporulating plates or liquid culture, and sprayed onto the leaves of 2 week old plants, at an appropriate concentration. The

inoculated plants are placed in a high humidity mistchamber for 2-3 days and subsequently moved to a growth chamber. The percent diseased leaf area is determined over the next 3 weeks and the presence or absence of pycnidia is noted.

3. Septoria tritici assay

S. tritici causes a leaf blotch disease on wheat. For the disease assays, spores are isolated from sporulating plates or liquid cultures, and sprayed, at an appropriate concentration, onto leaves of 1-2 week old wheat plants. The inoculated plants are placed in a high humidity mistchamber for 3 days and subsequently moved to a growth chamber. The percent diseased leaf area is determined 2-3 weeks after inoculation and the presence and absence of pycnidia is noted.

4. Leaf disc assays

The whole-plant disease assays described above can also be performed on leaf discs that are placed in moist petridishes.

5. Fusarium pouch assay

This assay is similar to the F. moniliforme and P. aphinadermata assays for maize described above. Spores from F. culmorum are harvested from plates as described for B. maydis. Perforated filter paper is folded, placed in a pouch, and surface sterilized wheat seeds are placed in the fold, such that the emerging roots grow through the perforations to the bottom of the pouch. A 12 ml spore suspension of F. culmorum in Hewitt's medium (a nutrient medium to allow the wheat seeds to germinate and grow) is placed in the bottom of the pouch and the pouch is incubated in a growth chamber at 10-15°C for about 1 week, after which the incubation is continued at 15-18°C. About four days later rootlength and rootbrowning is scored.

6. Powdery mildew leaf assay

Erysiphe graminis f. sp.tritici causes powdery mildew on wheat leaves. It is an obligate biotroph and hence inoculum is maintained on wheat leaves. Plants for disease testing are grown to 12-14 days of age, 2.5 cm sections are cut from the leaves and placed on agar plates containing 50 mg/ml benzimidazole. Spores collected from infected leaves are used to inoculate the leaf pieces on the agar plate, at an appropriate concentration. Next, the plates are incubated at 17°C under low light conditions. Disease symptoms on the leaf pieces are evaluated approximately 10 days after inoculation.

Example 11: Human Lactoferricin (Lacto H)

An antibacterial and antifungal peptide has also been isolated from human lactoferrin, referred to as lactoferricin H. This peptide corresponds to aminoacids 1-33 of the mature protein. A synthetic gene is generated that codes for the lactoferricin H peptide (SEQ ID NO:2), with codons optimized for expression in maize (SEQ ID NO:5):

```
 G   R   R   R   R   S   V   Q   W   C   A
GGC-CGC-CGC-CGC-CGC-AGC-GTG-CAG-TGG-TGC-GCC-

 V   S   Q   P   E   A   T   K   C   F   Q
GTG-AGC-CAG-CCC-GAG-GCC-ACC-AAG-TGC-TTC-CAG-

 W   Q   R   N   M   R   K   V   R   G   P
TGG-CAG-CGC-AAC-ATG-CGC-AAG-GTG-CGC-GGC-CCC
```

To allow transcription and translation of this gene sequence, a methionine start codon and a stopcodon are added. In addition, the ACC sequence from the Kozak consensus sequence is added to the beginning of the sequence, to improve the likelihood of efficient translation. This generates the Met-Lactoferricin H gene sequence as shown below, with the start and stop codons underlined.

5'-ACCATGGGCCGCCGCCGCCGCAGCGTGCAGTGGTGCGCCGTGAGCCAGCCCGAGGCCACCAAG-
TGCTTCCAGTGGCAGCGCAACATGCGCAAGGTGCGCGGCCCCTAG-3' (SEQ ID NO:6)

This gene sequence is expressed in plant cells under the control of an appropriate promoter, using methods analogous to those descibed in the preceeding examples for lactoferricin B.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: NOVARTIS AG
(B) STREET: Schwarzwaldallee 215
(C) CITY: Basel
(E) COUNTRY: Switzerland
(F) POSTAL CODE (ZIP): 4058
(G) TELEPHONE: +4161 324 11 11
(H) TELEFAX: + 4161 322 75 32

(ii) TITLE OF INVENTION: Peptide with inhibitory activity towards plant pathogenic fungi

(iii) NUMBER OF SEQUENCES: 46

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bovine Lactoferricin

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Phe Lys Cys Arg Arg Trp Gln Trp Arg Met Lys Lys Leu Gly Ala Pro
1 5 10 15

Ser Ile Thr Cys Val Arg Arg Ala Phe
20 25

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human Lactoferricin

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Gly Arg Arg Arg Arg Ser Val Gln Trp Cys Ala Val Ser Gln Pro Glu
1 5 10 15

Ala Thr Lys Cys Phe Gln Trp Gln Arg Asn Met Arg Lys Val Arg Gly
20 25 30

Pro

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Plant optimized bovine lactoferricin gene

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TTCAAGTGCC GCCGCTGGCA GTGGCGCATG AAGAAGCTGG GCGCCCCCAG CATCACCTGC 60

GTGCGCAGGG CCTTC 75

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 78 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Plant optimized bovine lactoferricin gene

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TTCAAGTGCC GCCGCTGGCA GTGGCGCATG AAGAAGCTGG GCGCCCCCAG CATCACCTGC 60

GTGCGCAGGG CCTTCTAA 78

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 99 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Plant optimized human lactoferricin gene

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GGCCGCCGCC GCCGCAGCGT GCAGTGGTGC GCCGTGAGCC AGCCCGAGGC CACCAAGTGC 60

TTCCAGTGGC AGCGCAACAT GCGCAAGGTG CGCGGCCCC 99

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 108 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Plant optimized human lactofericin gene

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

ACCATGGGCC GCCGCCGCCG CAGCGTGCAG TGGTGCGCCG TGAGCCAGCC CGAGGCCACC 60

AAGTGCTTCC AGTGGCAGCG CAACATGCGC AAGGTGCGCG GCCCCTAG 108

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 91 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Oligo 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GATCCACCAT GTTCAAGTGC CGCCGCTGGC AGTGGCGCAT GAAGAAGCTG GGCGCCCCCA 60

GCATCACCTG CGTGCGCAGG GCCTTCTAAG C 91

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 91 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Oligo 2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GGCCGCTTAG AAGGCCCTGC GCACGCAGGT GATGCTGGGG GCGCCCAGCT TCTTCATGCG 60

CCACTGCCAG CGGCGGCACT TGAACATGGT G 91

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Oligo 1-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

AGTAGGATCC ACCATGTTCA AGTGCC 26

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Oligo 2-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

TACTGCGGCC GCTTAGAAGG CCCTGCGC 28

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 88 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Oligo 3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GATCCACCAT GGCACCGAGG CTAGCGTGCC TCCTAGCTCT GGCCATGGCA GCCATCGTCG 60

TGGCGCCATG CACGGCCTTC AAGTGCCG 88

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 88 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Oligo 4

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GGCCGCTTAG AAGGCCCTGC GCACGCAGGT GATGCTGGGG GCGCCCAGCT TCTTCATGCG 60

CCACTGCCAG CGGCGGCACT TGAAGGCC 88

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Oligo 3-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

AGTAGGATCC ACCATGGCAC CGAGGCTAG 29

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Oligo 5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

AGTACCATCG TCGTGGCGCC ATGCACGGCC CAGTTCAAG 39

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Oligo 6

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

AGTAGCGGCC GCTTAGATGG CCTCGGCGAA CACGAAGGCC CTGCGCACGC A 51

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: ATP-5'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

AGTAGGATCC ACCATGGCTT CCTCTATGCT C 31

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: ATP1-3'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GCAGTTAACT CTTCCGCCGT T 21

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: ATP-LF 5'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

GTAGGATCCA CCATGGCT 18

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: ATP-LFB 5'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

GGCGGAAGAG TTAACTGCTT CAAGTGCCGC CGCTGG 36

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: ATP-LFB 3'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

CCAGCGGCGG CACTTGAAGC AGTTAACTCT TCCGCC 36

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: ATP2-3'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

CTGCATGCAG TTGACGCGAC CACCGGAATC GGTAAGGTCA GG 42

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: ATP3-3'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

GCAGTTGACG CGACCACCGG AATCGGTAAG GTCAGG 36

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: ATP2-LFB 5'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

CGCGTCAACT GCATGCAGTT CAAGTGCCGC CGCTGG 36

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: ATP3-LFB 5'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

GGTGGTCGCG TCAACTGCTT CAAGTGCCGC CGCTGG 36

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: ATP2-LFB 3'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

CCAGCGGCGG CACTTGAACT GCATGCAGTT GACGCG 36

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: ATP3-LFB 3'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

CCAGCGGCGG CACTTGAAGC AGTTGACGCG ACCACC 36

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: MTP-5'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

AGTAGGATCC ACCATGGCGC CCACCGTGAT G 31

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: MTP1-3'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

GCACCGGATT CTTCCGCCGT T 21

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: MTP2-3'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

CTGCATGCAC CGTATGCGAC CACCGTCCGT CGACAGCGGC GG 42

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: MTP3-3'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

GCACCGTATG CGACCACCGT CCGTCGACAG CGGCGG 36

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: MTP-LF 5'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

GTAGGATCCA CCATGGCG 18

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: MTP-LF 3'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

GCGGCCGCTT AGAAGGC 17

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: MTP1-LFB 5'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

GGCGGAAGAA TCCGGTGCTT CAAGTGCCGC CGCTGG 36

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: MTP2-LFB 5'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

CGCATACGGT GCATGCAGTT CAAGTGCCGC CGCTGG 36

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: MTP3-LFB 5'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

GGTGGTCGCA TACGGTGCTT CAAGTGCCGC CGCTGG 36

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: MTF-LFB 3'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

CCAGCGGCGG CACTTGAAGC ACCGGATTCT TCCGCC 36

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: MTF2-LFB 3'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

CCAGCGGCGG CACTTGAACT GCATGCACCG TATGCG 36

(2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: MTF3-LFB 3'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

CCAGCGGCGG CACTTGAAGC ACCGTATGCG ACCACC 36

(2) INFORMATION FOR SEQ ID NO: 39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Primer 176

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

TTCCCCAACC TCGTGTTGTT C 21

(2) INFORMATION FOR SEQ ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Primer 179

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

CCAAATGTTT GAACGATCGC G 21

(2) INFORMATION FOR SEQ ID NO: 41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Primer 177

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

CACAACCAGA TCTCCCCCAA A 21

(2) INFORMATION FOR SEQ ID NO: 42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Primer 180

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

AAATTCGCGG CCGCTTAGAA G 21

(2) INFORMATION FOR SEQ ID NO: 43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43:

AGTAGGATCC ATGAAAAAGC CTGAACTC 28

(2) INFORMATION FOR SEQ ID NO: 44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44:

TACTGGATCC CTATTCCTTT GCCCTC 26

(2) INFORMATION FOR SEQ ID NO: 45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45:

TATCTCTCTC TATAAGGATC CATGGTCACC 30

(2) INFORMATION FOR SEQ ID NO: 46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46:

TACTGGATCC TCAGTACACA GTCCTGCC 28

What is claimed is:

1. A recombinant DNA molecule comprising in operative sequence:
   (a) a promoter which functions in plant cells to cause the production of an RNA sequence;
   (b) a structural coding sequence that encodes for production of lactoferricin; and
   (c) a 3' non-translated region which functions in plant cells to cause the addition of polyadenylate nucleotides to the 3' end of the RNA sequence.

2. The DNA molecule of Claim 1 wherein the promoter is the ubiquitin promoter and the 3' non-translated region is the nopaline synthase transcriptional terminator from *Agrobacterium tumifaciens*.

3. The DNA molecule of claim 1 or 2 wherein the lactoferricin is lactoferricin B.

4. A method of producing transgenic, disease resistant plants, comprising the steps of:
   (a) inserting into the genome of a plant cell recombinant DNA according to claim 1;
   (b) obtaining transformed plant cells, and
   (c) regenerating from the transformed plant cells transgenic plants which express lactoferricin in an amount effective to reduce damage from disease.

5. The method of claim 4 wherein said plant is selected from maize, wheat, and sugar beet.

6. The method of claim 4 or 5 wherein the lactoferricin is lactoferricin B.

7. A plant comprising cells which express lactoferricin.

8. A plant according to claim 7 having stably integrated in its genome recombinant DNA according to claim 1.

9. A plant according to claim 7 or 8 selected from maize, wheat, or sugar beet.

10. Seed which when germinated and cultivated will produce a plant according to claims 7, 8 or 9.

11. Seed according to claim 10 which is packaged or coated.

12. A plant or seed according to claims 7-11 wherein the lactoferricin is lactoferricin B.

13. Use of lactoferricin in the control of plant pathogens.

14. A method of controlling plant pathogens comprising exposing said pathogens to a plant according to claim 7.

15. An agricultural method, wherein the transgenic plant according to claim 7 or the progeny thereof is used to control plant pathogens.

16. A commercial bag containing seed according to claim 10.

Fig. 1

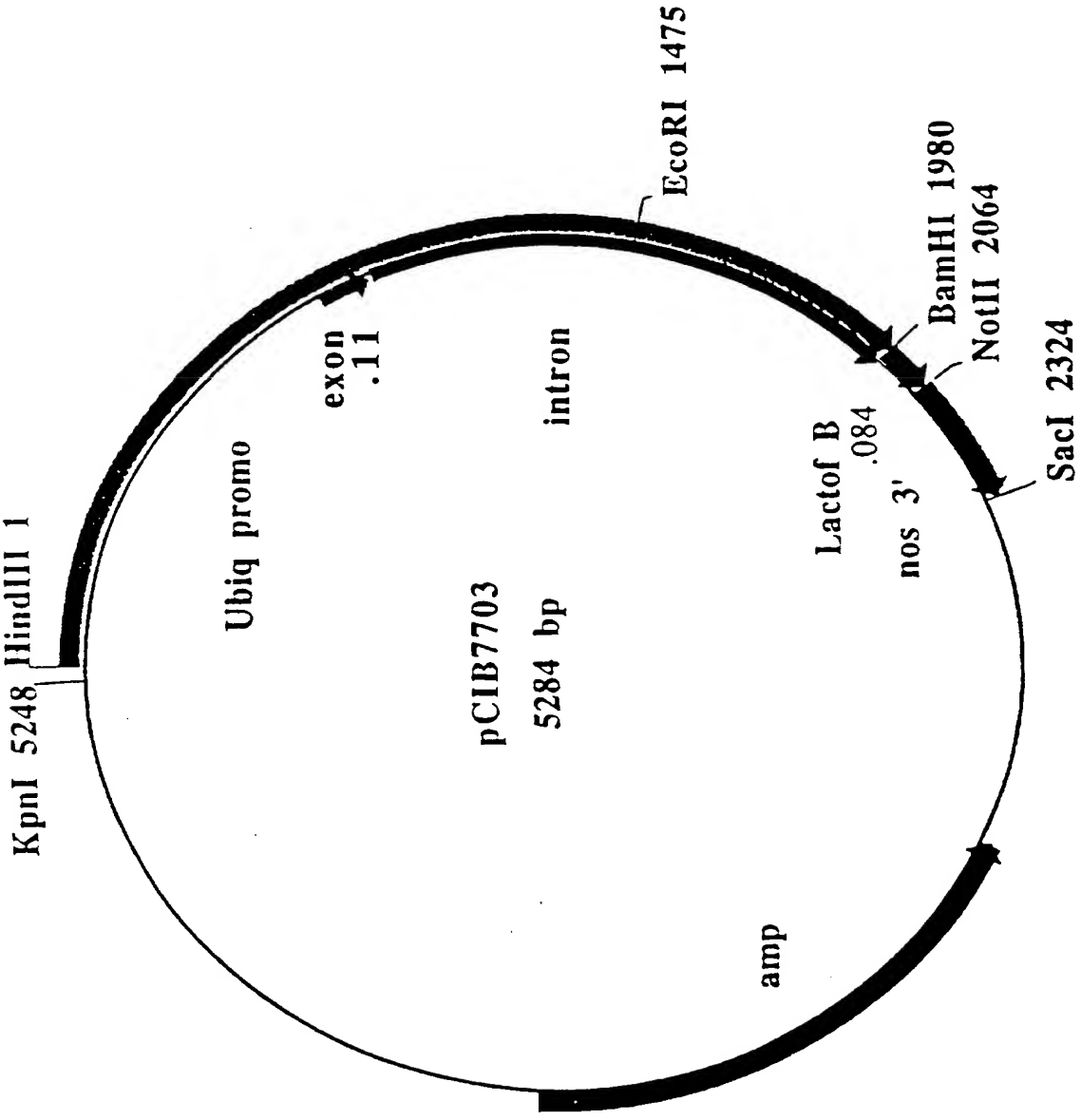
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Fig. 2

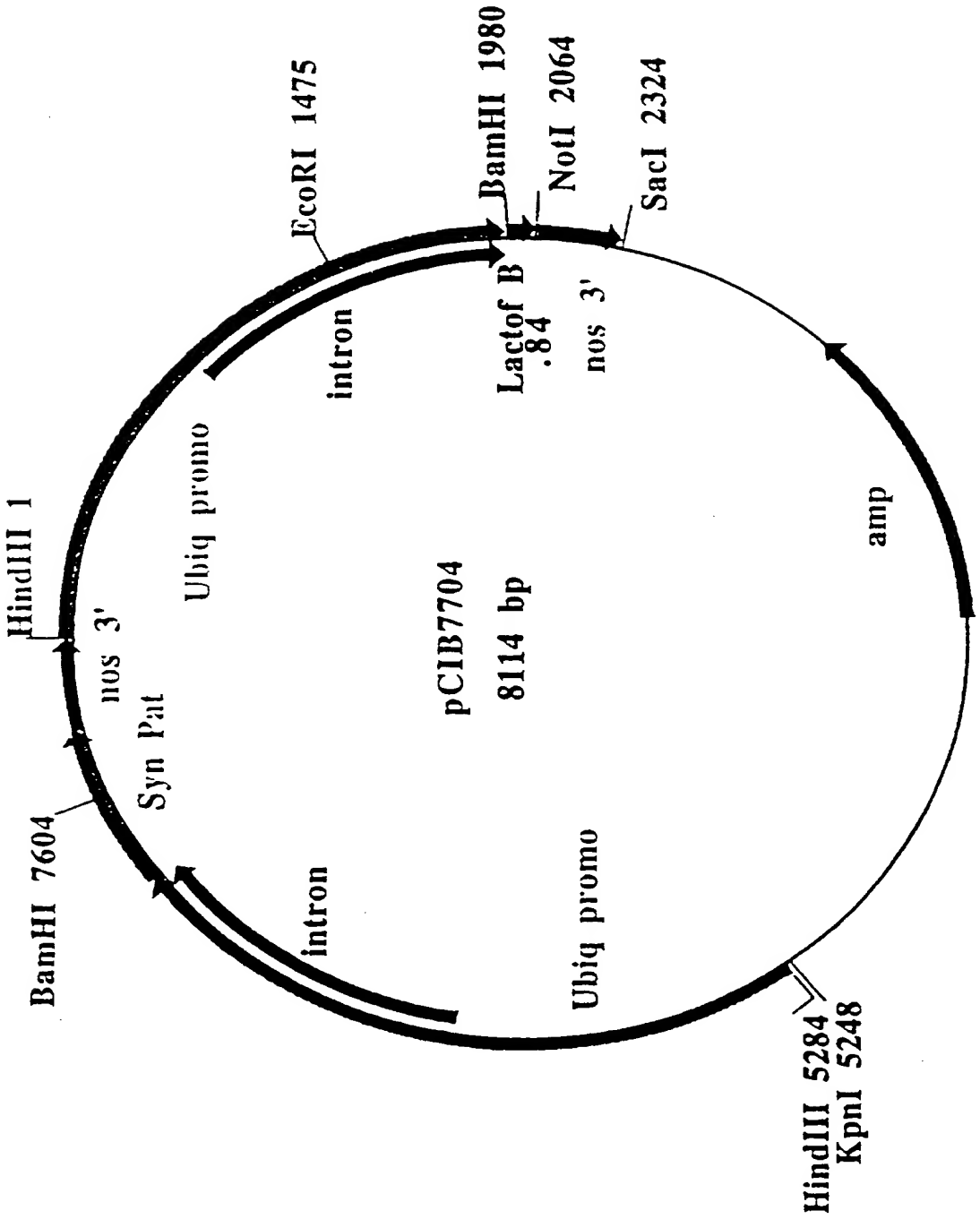


Fig. 3

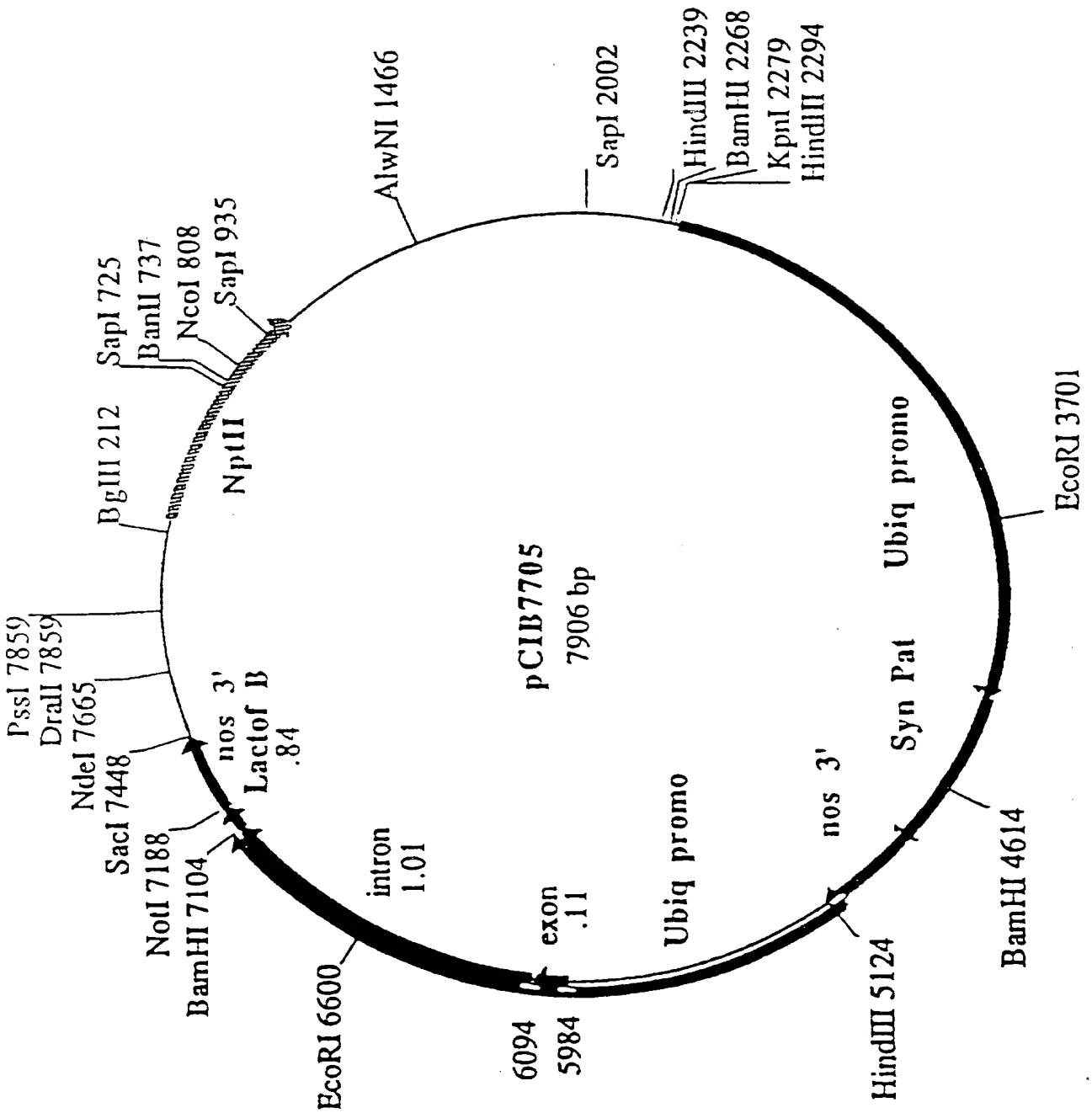
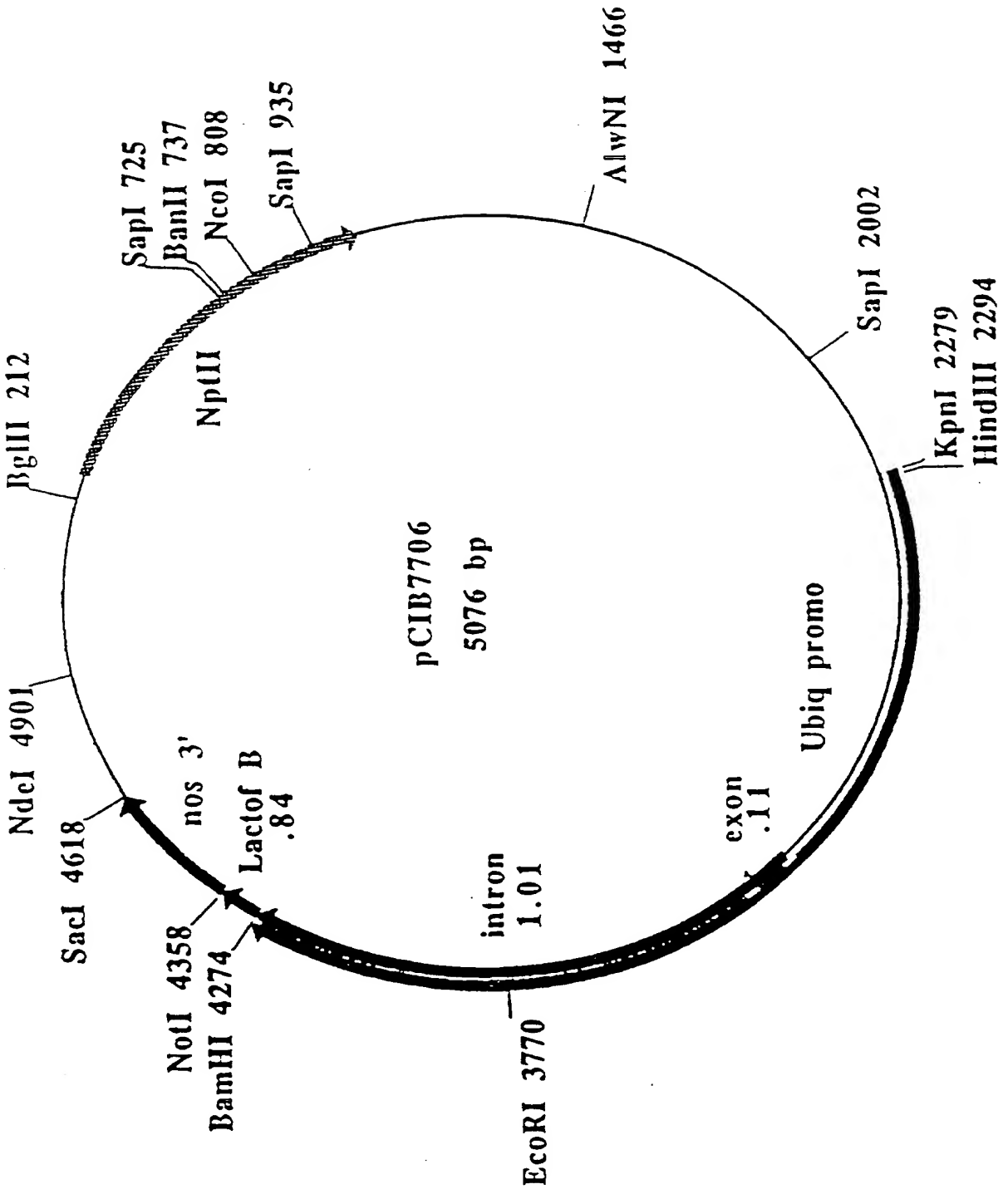


Fig. 4



INTERNATIONAL SEARCH REPORT

Inter· ɔnal Application No
PCT/EP 97/04438

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N15/12 A01N63/02 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01N A01H C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZHANG, Z., ET AL.: "Gene transfer for enhancing plant disease-resistance to bacterial pathogens" BIOTECHABS DATABASE, AN 96-00905, XP002048440 see abstract & HORTSCIENCE, vol. 30, no. 4, 1995, page 788	1,13
Y		2-4,6,7, 10-12, 14-16

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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1

Date of the actual completion of the international search	Date of mailing of the international search report
5 December 1997	29/12/1997

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Maddox, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	BELLAMY, W., ET AL.: "Identification of the bactericidal domain of lactoferrin" BIOCHIM. BIOPHYS. ACTA, vol. 1121, 1992, pages 130-136, XP002049260 see the whole document	2-4,6,7, 10-12, 14-16
X	ZHANG, ZHANYUAN 'PH.D.! ET AL: "DEVELOPMENT OF TRANSGENIC PLANTS WITH NON-PLANT ANTIBACTERIAL PROTEIN GENES FOR RESISTANCE TO BACTERIAL PATHOGENS (LACTOFERRIN, NICOTIANA TABACUM, AGROBACTERIUM TUMEFACIENS)" DISSERTATION ABSTRACTS DATABASE AN 97:3204 & DISSERTATION ABSTRACTS INTERNATIONAL, (1996) VOL. 57, NO. 7B, P. 4124. ORDER NO.: AAR9637086. 185 PAGES., XP002048439 see the whole document	1,13
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